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THE GROWTH AND INORGANIC NUTRITION OF SCENEDESMUS OBLIQUUS IN MASS CULTURE[1,2,3]

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Current interest in the possible commercial use of algae resulting from the investigations of Spoehr and Milner (30), Spoehr (29), Coombs (7), and Burlew (6), as well as continued recognition of their value as tools in many fields of research, has emphasized the desirability of an understanding of the factors which may limit algal growth under conditions of mass culture. The first requirement is a culture apparatus which can maintain constant light and temperature. The second demand is for a technique which will provide a constant supply of nutrients in the medium over an extended period of time. The investigation of other factors affecting the photosynthetic efficiency of mass cultures can proceed only after these requisites are satisfied. The bulk of the earlier work on growth and inorganic nutrition of algae prior to 1942, reviewed by Bold (4), has been concerned primarily with the growth of algae in small pure cultures which were allowed to grow to maturity. The adequacy of the initial medium supplied such "batch" or discontinuous cultures is determined by the amount of growth resulting during a given period of time without regard to the progressive environmental changes in the medium. However, Ketchum and Redfield (13) developed an apparatus using an artificially illuminated carboy for the continuous culture of marine diatoms to test the principle of the optimum catch delineated by Hjort, Jahn, and Ottestatt (11). This technique was modified by Scott (27) in determining the growth and inorganic content of Chlorella under varying nutrient concentrations of magnesium, calcium, potassium, and sodium. Myers and Clark (19) also achieved a continuous system approaching the steady state by controlling photoelectrically the dilution of the culture with fresh medium which serves to replace the inorganic nutrients, to maintain a constant population density, and to measure growth by overflow. This technique has been used to determine the effectiveness of various inorganic and organic nutrient solutions on growth. The present state of our knowledge of inorganic nutrition derived from both continuous and discontinuous culture of the algae has been reviewed by Myers (18) and Krauss (15). The extension and application of this knowledge to produce optimum yields from open cultures has uncovered specific problems, demanding further investigation for which mass cultures permitting large and frequent samples are uniquely suited. The study reported in this paper was designed to determine the inorganic requirements and growth characteristics of *Scenedesmus obliquus* (Turp.) Kütz. in a continuous mass culture. It is the initial step in an effort to elucidate all factors limiting the growth of algae in mass cultures.

MATERIALS AND METHODS

APPARATUS: The culture apparatus is diagrammed in figure 1. The algae were grown in glass-covered, polyethylene-lined, white, plywood vats having an illuminated surface area of 2 m^2 and a depth of 20 cm (A). In all experiments the vats were filled to a depth of 15 cm giving cultures of 300 liters. Motors (B) driving stainless steel propellers mounted in diagonal corners of the vat provided rapid circulation to prevent sedimentation. Water for the cultures heated to 70°C was passed through a cooling coil in a cold water bath (I) and then through an ion exchange column (J). Such water with less than 2 ppm of electrolyte (as NaCl) was essentially free of contaminating organisms. A mixture of 5 % CO_2-in-air metered by flow meters (CC) was supplied to the culture through a porous carbon pipe (C) at a rate of 50 l/hr.

Each vat was illuminated by a battery of lights containing 16 fluorescent and 12 incandescent lights mounted on a reflector (S) which was lowered over the vats during culture. The value of a balanced combination of incandescent and fluorescent lamps has been discussed by Parker and Borthwick (22). Such a source gives constant light of as high an intensity and with a spectral distribution as near to that of sunlight as is feasible with artificial sources. Ballasts (R) for the fluorescent tubes were mounted outside the chamber to exclude heat. The light intensities read from a Weston Illumination Meter (Model 756) are plotted in figure 2. After burning the bulbs

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[2] The investigations reported here were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Maryland (Nonr 556(00)). Additional support was made possible through the cooperation of the Carnegie Institution of Washington.

[3] Paper No. A403, Contribution No. 2431 of the Maryland Agricultural Experiment Station (Dept. of Botany).

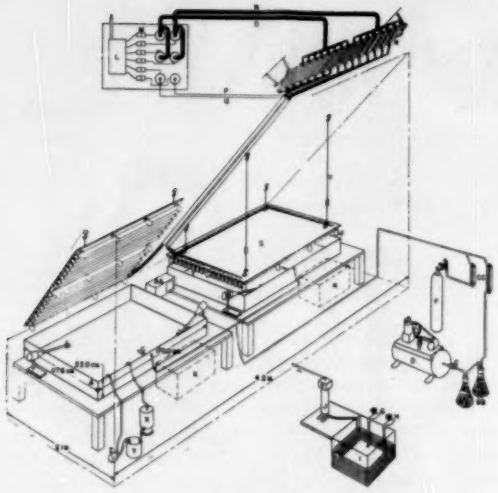


FIG. 1. Diagram of the controlled environment chamber and algal culture apparatus: white, polyethylene-lined, plywood vat (A); 1/30 HP motor driving stainless steel propeller at 1600 RPM (B); porous carbon delivery pipe for CO_2 (C); air compressor (D); diaphragm valve (E); CO_2 cylinder (F); cold water (G); hot water (H); cold water bath (I); cation-anion exchange column (J); air conditioners (K); main light control panel (L); individual bank switches (M); power lines to fluorescent batteries (N) (O); power lines to incandescent batteries (P) (Q); 430 milliampere ballasts (R); top view, right reflector board in operating position (S); 4500° white 72-T-12 General Electric fluorescent tubes, combined power 9600 watts per panel (T); incandescent lamps, frosted, combined power 7200 watts per panel (U); chain and turnbuckle support (V); solid-basket type chemical centrifuge (X); reservoir (Y); centrifugal pump (Z); hygrothermograph (AA); glass wool and cotton air filters (BB); variable-area, float-type flow meters (CC).

200 hours to remove the abnormally high initial intensity, the maximum illuminance at the center of the culture surface was 1700 fc, averaging 1,000 fc for the entire surface. Irradiance at the same level reached 0.17 gm cal/cm^2 × sec and averaged 0.12 gm cal/cm^2 × sec as measured by a General Electric radiation meter Type DW-60. Temperature was thermostatically controlled by means of two air conditioners (K) mounted in the wall of the chamber. Ambient temperatures of 22°C were required to hold the temperatures in the cultures to the desired 28°C. Temperature was recorded on a hygrothermograph (AA). Harvesting of the cells from the culture was effected by siphoning the culture through a continuously operating stainless steel centrifuge (X). The supernatant medium discharged to a porcelain reservoir from which it was returned to the vat by means of a rubber centrifugal pump (Z).

GROWTH: A strain of *Scenedesmus obliquus* designated "WH 50" obtained from the Biological Laboratory at Woods Hole in 1950 and maintained on agar slants was used in all experiments. For inocula the algae were subcultured in a 1-liter Erlenmeyer flask supplied with 5 % CO_2-in-air plus a basal nutrient medium. A total packed cell volume of 0.01 ml added to 300 l, giving the very low concentration of 1,000 cells per ml, was the initial inoculation for each experiment.

Growth in the cultures was followed by three methods: cell count using a hemacytometer, packed cell volume per 100 ml (hereafter referred to as pcv) measured by centrifugation in Goetz phosphorous tubes according to the method of Ketchum and Redfield (14), and dry weight of packed cells resuspended in distilled water and transferred to tared aluminum cups for drying to constant weight in a vacuum oven at 40°C.

The bacterial counts were made from plates of nutrient agar incubated for 48 hours at 30°C according to the method of Scott (28).

ANALYTICAL METHODS: Samples of the culture medium and of the cells were taken every 12 hours. The cell samples were dried in vacuo and ground in a Wiley Mill to pass a 40-mesh screen prior to reweighing for ashing. The samples of the medium were centrifuged free of cells and stabilized with 3 ppm of mercury as bichloride to prevent biological modification of the solution during the interval between sampling and analysis. Nitrate N in the medium was determined by nitration of 4-hydroxy-1,3-dimethylbenzene, as modified from the method of von Alten et al (32). The 4-hydroxy-5-nitro-1,3-dimethylbenzene was distilled, collected in 12 *N* NaOH, brought to volume, and the transmission read in a Fisher electrophotometer. In the medium, nitrate and ammoniacal N, designated "total nitrogen," were measured by the Devarda method as given in AOAC (16). The cells were analyzed for total N by the semi-micro adaption of the Kjeldahl method of Ranker (24) using copper and selenium catalysts and the Kemmerer-Hallett distillation unit. By this method loss of nitrate nitrogen is prevented by addition of salicylic acid. Phosphate in the medium was determined colorimetrically by the ammonium molyb-

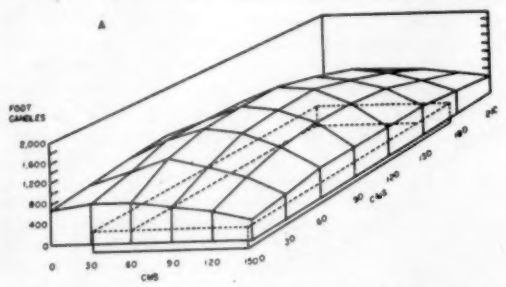


FIG. 2. Diagrammatic representation, expressed in fc, of illuminance at the surface of a culture from a battery of fluorescent and incandescent lamps.

date method as given by Lindner (17). In this analysis greater precision was obtained by omitting the sodium hydroxide normally required to prevent the excess acidity found in ashed samples dissolved in hydrochloric acid. Phosphate in the dried cells was analyzed by the method in AOAC (16). The sulfate content of the medium was determined by the semimicro method of Reitemeier (25) involving the titration of benzedine sulfate with sodium hydroxide. The cells were ashed with magnesium nitrate after which sulfate was precipitated as $BaCl_2$ according to the gravimetric procedure of AOAC (16). Potassium analyses were obtained for both the medium and plant material by flame photometry in a Perkin Elmer Flame Photometer (Model 52-C), using an internal lithium standard for all samples. Calcium in the medium was also determined by means of the flame photometer. The analyses of the cells were carried out by precipitation of calcium as calcium oxalate as given by Blasdale (3). Magnesium analyses of the medium were made with the Titan Yellow method of Peech and English (23). However this method as well as the soap titration technique of Burgess and Breazeale (5) proved to be highly erratic and the analyses showing no absorption of the magnesium ion from the solution are considered unsatisfactory. The gravimetric analyses of the cells by precipitation of magnesium as magnesium-ammonium-phosphate (16) proved entirely satisfactory and reproducible. The chloride concentration in the medium was determined by the silver nitrate titration as given by AOAC (16). Bicarbonate and carbonate values were obtained by titrating the cell free medium to the phenolphthalein and methyl orange end points immediately after centrifugation of the samples. The method employed was that of AOAC (16). A Model H Beckman pH meter was used for all determinations of hydrogen ion concentration.

Results and Discussion

The first five cultures were grown in Detmer's medium having the following composition: 100.0 gm $Ca(NO_3)_2 \cdot 4\,H_2O$; 25.0 gm KCl; 25.0 gm $MgSO_4 \cdot 7\,H_2O$; 25.0 gm KH_2PO_4; 0.2 gm $FeCl_3$; water to make 300 l. This medium has been successfully employed for the culture of many species of algae and it was used for introductory studies, first without the addition of micronutrients and in later experiments with micronutrients supplied.

In view of the work by Osterlind (20, 21), which indicated that certain algae could utilize bicarbonates as readily as dissolved CO_2 for a carbon source, the first experiment was run to determine if the periodic addition of pure CO_2 would serve as an adequate supply of CO_2. It was considered possible that the intermittent addition of pure CO_2 would form sufficient bicarbonates to supply the cells adequately during the periods when gas was not being delivered. If this were true the necessity of maintaining a continuous supply of 5 % CO_2-in-air would be removed. Therefore pure CO_2 was bubbled through the culture for one hour during each 24-hour period. In 21 days the culture had grown from an inoculum of 1,000 cells/ml to a concentration of 21,000,000 cells/ml indicating that considerable growth could be obtained in this fashion. Of greater interest was the effect of the one hour of bubbling of CO_2 on the pH in the solution. During the course of growth there is a rise in the pH of all nutrient solutions supplying nitrogen in the form of nitrate as shown by Urham (31), Gerloff et al (10), Fowden (9), and others. At the end of the 10th day the pH of the first culture had risen from 6.2 to 10.0. Immediately after one hour of bubbling carbon dioxide, however, the pH dropped to 6.5 from which level it climbed rapidly to 10.5 indicating an efficient removal from solution of either dissolved CO_2 or buffering bicarbonate ions. This fluctuation in pH was observed repeatedly during the culture. In a second culture a continuous mixture of 5 % CO_2-in-air was supplied delivering a constant supply of carbon and maintaining a steady pH level. This technique resulted in more rapid growth and was adopted for all subsequent cultures. Nevertheless these more satisfactory results obtained with the classical 5 % CO_2-in-air mixture do not preclude the effective use of frequent intermittent injections of pure CO_2. Davis (8) has pointed out that maximum growth with Chlorella could be obtained with a CO_2 concentration slightly above 0.03 % as long as rapid dispersal of the gas maintained a saturated condition throughout the culture. The problem of rapid dispersal of pure CO_2 to prevent toxic concentrations, however, can be most easily handled by advance dilution with air. In spite of the preferential removal of inorganic anions the pH of the second culture rose only to pH 8.0. The conductivity, reflecting both cation and anion uptake, was reduced from the initial 65.0 ($K \times 10^5$) mhos to 44.0 ($K \times 10^5$) mhos by the 15th day.

In order to determine the rate of N uptake from the medium, analyses for N content were made during 18 days of growth in a third culture. The nitrate content of the medium declined from the initial 175 ppm to 0 ppm by the 14th day. Growth nevertheless continued indicating subsequent sharing of previously absorbed N by daughter cells. Analyses for each of the other major nutrients demonstrated that only N was exhausted early in the period of culture. Nitrogen could presumably be replaced by periodic additions of dilute HNO_3 thus leaving only one major limiting factor to be controlled—the reduction of light available per cell unit resulting from the increase in cell concentration.

Cultures 4 and 5 were run to investigate the effects of both a supplemental supply of N and the removal of the daily cell increment to prevent progressive reduction in light per unit cell. On the eighth day after inoculation at a concentration of 1.3×10^6 cells/ml centrifugation was begun in culture five. At this point the populations of the two cultures were similar. The nitrate level in both cultures was maintained by periodic addition of dilute HNO_3.

TABLE I

DRY CELL WEIGHT AND INORGANIC ANALYSES OF THE MEDIUM DURING GROWTH OF SCENEDESMUS OBLIQUUS IN CULTURE FOUR

SAMPLE DAYS AFTER INOCULATION	MILLI-EQUIVALENTS PER LITER								pH	GM DRY WEIGHT PER 300 L
	K	Ca	NO_3	PO_4	SO_4	Cl	HCO_3	CO_3		
0	1.74	2.80	2.65	1.80	0.67	1.17	0.17	0.0	5.0	00.0
2	1.79	2.80	2.81	1.82	0.66	1.20	0.17	0.0	5.1	...
4	1.76	2.80	2.76	1.80	0.70	1.17	0.14	0.0	5.4	12.6
6	1.76	2.65	2.27	1.64	0.67	1.20	0.75	0.0	6.9	27.2
8	1.81	2.10	1.65	1.66	0.61	1.20	1.31	0.0	7.2	45.9
10*	1.76	2.55	2.84	1.24	0.59	1.19	0.17	0.0	4.8	61.2
12	1.69	2.45	2.45	1.14	0.54	1.18	0.46	0.0	6.7	63.9
14	1.69	1.80	1.74	0.56	0.49	1.20	1.20	0.0	7.6	105.6
16	1.61	2.35	1.14	0.80	0.33	1.19	1.93	0.0	7.3	141.6
18	1.51	1.70	0.46	0.56	0.30	1.18	2.50	0.0	7.5	165.6
20	1.51	1.65	0.00	0.28	0.28	1.17	3.00	0.0	8.4	209.9

* By the addition of dilute HNO_3 the nitrate level was increased 1.4 mEq/l on the tenth day.

However the daily removal of the cell increment from culture 5 resulted in no increase in the total dry weight of cells produced by culture 5 nor was there a major increase in weight in either culture. A factor other than light or nitrogen had become limiting. The reduction in growth could either be attributed to the accumulation of toxic material or the depletion of a required nutrient. To test whether certain micronutrients may have become limiting, manganese, boron, and zinc in the concentrations used by Craig and Trelease, given by Bold (4), were added to both cultures on the twelfth day. Growth immediately resumed. During the first two subsequent days the yield was greater in the centrifuged culture; on the succeeding days this total yield was matched and then exceeded by the non-centrifuged culture suggesting subtraction, by daily harvest, of micronutrients which might otherwise have been shared by subsequent generations in culture 5. In neither culture was the higher growth rate prolonged. Clearly micronutrients introduced only as impurities in the macronutrient salts are insufficient to support continued growth in mass cultures, nor can rapid growth rates be sustained by the addition of only manganese, boron, and zinc. Even though water may have appeared to supply adequate micronutrients in innumerable cultures in the past the effect here obtained would render explicit conclusions based on such cultures open to question.

Inorganic analyses showing iron removal from the medium in culture 4 are given in table I. The Mg analyses using the Titan Yellow technique of Peech and English (23), which proved troublesome as a result of some interfering substance in the samples, are omitted. The 1.4 mEq of HNO_3 per liter added on the tenth day is reflected in the analyses. It is also reflected in the reduction of the pH to 4.2 and 4.8 rather than to the initial 5.0. The preferential uptake of anions, primarily nitrate, results in an increasing concentration of HCO_3^- which is reduced temporarily by the addition of the supplementary nitrate and readjustment of pH. The cell counts and weights showing lower concentrations of cells in culture 5 indicate some injury resulting from the sudden drop in pH. From the information delineating comparative rates of ion uptake from the medium, a nutrient solution was prepared which reflected more nearly the requirements of the algae for both major and minor nutrients. The formula adopted for subsequent cultures is given in table II.

Chlorine and calcium were omitted from the major nutrient complement. The concentration of major nutrients was increased by a factor of three except

TABLE II

INORGANIC MEDIUM FOR SCENEDESMUS OBLIQUUS

MACRONUTRIENTS

Cmpd.	*mEq/l*
KNO_3	9.9
$MgSO_4 \cdot 7\,H_2O$	2.0
KH_2PO_4	5.5

MICRONUTRIENTS *

Cmpd.	*Stock solution gm/l*	*0.1 ml/l gives ppm of the metal*
H_2BO_4	2.860	0.05
$MnCl_2 \cdot 4\,H_2O$	1.810	0.05
$ZnSO_4 \cdot 7\,H_2O$	0.222	0.005
$CuSO_4 \cdot 5\,H_2O$	0.079	0.002
MoO_3	0.015	0.001
$Ca(NO_3)_2 \cdot 4\,H_2O$	59.000	1.00
$CoCl_2 \cdot 6\,H_2O$	0.04	0.001

IRON SOLUTION **

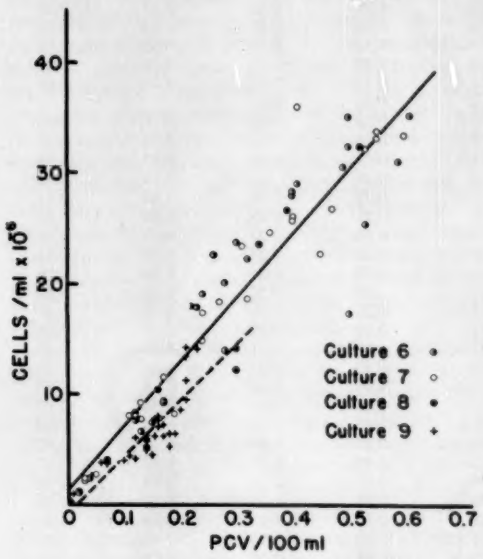
Cmpd.	*Stock solution gm/l*	*1 ml/l gives ppm of Fe*
Ferric citrate ($FeC_6H_5O_7 \cdot 3\,H_2O$)	5.30	1.0
Citric acid	5.30	0.0

* These salts are dissolved in 1 liter 0.1% HCl. Modified from Arnon (2).

** Modified from Rodhe (25).

for potassium which was present in excess. A more complete micronutrient solution modified from Arnon (2) was included. Boron, cobalt, and molybdenum were also added although the essentiality of these ions has not yet been demonstrated for the algae. The pH of the medium was adjusted to 7 by the addition of 1.6 ml of 5 % KOH per liter which gave a total value for potassium of 13.1 mEq/l. The adoption of this medium supplied succeeding cultures with nutrients more closely related to their quantitative and qualitative requirements than Detmer's solution and provided a starting point for a more detailed study of nutrient supply.

in earlier experiments. The relationship between packed cell volume (pcv) and dry weight, and between pcv and cell count, are given in figures 3 and 4. The regression line includes data from culture 8 grown under similar conditions. Packed cell volume may be converted to dry weight in gm/l by the equations for regression. Though pcv and dry weight are demonstrated to be more reliable indices for most of the growth period, cell counts during the early interval of low population are essential. During this period the pcv and dry weight are too small to be measured with reasonable accuracy. Growth in cultures 6 and 7 is given in table III in terms of pcv. On the twentieth



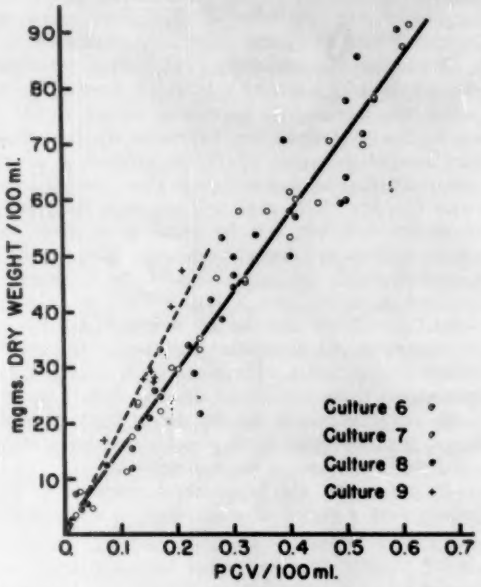


FIG. 3 (*left*). The quantitative relationship between the cell count and the packed cell volume (pcv) of *Scenedesmus obliquus* for cultures 6, 7, and 8, solid line, grown in a complete medium, and for culture 9, dotted line, grown in a micronutrient deficient medium. The regression calculated for the cells in the complete medium: cells/ml. $\times 10^{-6} = 1.4 + (57.9)$ (pcv); in the deficient medium: cells/ml $\times 10^{-6} = -0.84 + (51.4)$ (pcv).

FIG. 4 (*right*). The quantitative relationship between the dry weight and the packed cell volume (pcv) of *Scenedesmus obliquus* for cultures 6, 7, and 8, solid line, grown in a complete medium, and for culture 9, dotted line, grown in a micronutrient deficient medium. The regression calculated for the cells in the complete medium: mg dry weight/100 ml = 1.7 + (141.7) (pcv); in the deficient medium: mg dry weight/100 ml = −0.22 + (201.6) (pcv).

For the quantitative estimation of growth in cultures employing the new medium, measurements other than cell count were desired. The difficulty in obtaining precision by the use of the haemacytometer is generally recognized. Not only are cell counts often characterized by a large standard deviation but the count itself necessarily reflects cell division rather than production of cell material unless accompanied by determination of cell size. Therefore two cultures designated 6 and 7 were grown, using as measures of growth, cell count, packed cell volume, and dry weight. The cultures grown simultaneously in vats A and B were identical in all respects thus providing duplication and a test of reproducibility not obtained

day after inoculation the pcv in each vat was reduced from 0.60 to 0.13 ml/100 ml. Growth after this harvest was similar in rate and quantity to that produced during the earlier period of 7 days when pcv increased from 0.13 to 0.45 ml/100 ml. The total dry weight produced in culture 6 was 390.9 gm compared to 386.1 gm in culture 7. It averaged 0.0483 gm/day $\times$ l in culture 6 and 0.0476 gm/day $\times$ l in culture 7, a difference of less than 1 %.

Examination of the growth curves and calculated growth rates in all of the cultures reveals an unusually high rate of growth during the early period from inoculation to a concentration of 2×10^5 cells/ml or a pcv of 0.04 ml/100 ml after which the rate is rapidly

reduced. However established, an accurate estimate of growth during this period is difficult because of the relatively small population. Using the division rate established by cell count in the formula:

$$\log_n \frac{C_2}{C_1} = \bar{K}(T_2 - T_1)$$

where C_1 is the concentration at time T, C_2 the concentration at a later time T_2, and using logs to the base 2 ($3.32 \times \log_{10}$), the reciprocal of the generation time can be plotted. Figure 5 gives the growth rate, $\bar{K}$, for cultures 6, 7, and 8. Though the peak rate of 4.72 $\log_2$ units may be high due to measurement error, the average rate for the first and second days is 3.93 $\log_2$ units and this may be considered nearly the maximum rate attainable under ideal conditions.

To secure the maximum yield from a population characterized by a growth rate which decreases as the population increases, a harvesting regime should be established to remove any increment to the population beyond the point where the product of growth rate multiplied by concentration gives the maximum value (11, 12). This value will not be at the point of maximum rate but will be found at a lower rate which, applied to a higher population base, results in a greater yield. In these cultures the maximum is attained when the pcv is 0.10 ml/100 ml, the cell count 7.5×10^6/ml, and the dry weight 15 mg/100 ml. At this point, 4.5 days after inoculation, the growth rate is 1.0 $\log_2$ units. Therefore if this rate could be maintained (light is the only limiting factor) the culture should produce 45 gm dry weight every 24 hours. Essentially the same point is calculated using either cell count or pcv as an index of growth.

Before testing the hypothetical productivity of a culture over a period of many days, it was essential to know more closely how much N must be returned to the medium each day. It was also of interest to determine the total N content of cells grown under micronutrient deficiency. Cultures 8 and 9 were designed to give this information as well as to provide additional data concerning the relationships between pcv, cell count, and dry weight. The growth data are

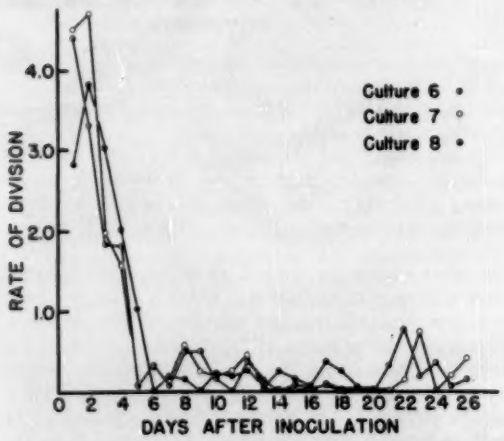


FIG. 5. Growth rates ($\bar{K}$) for *Scenedesmus obliquus* during cultures 6, 7, and 8 calculated from the formula $\log_2 \frac{C_2}{C_1} = \bar{K}(T_2 - T_1)$. The term, $\bar{K}$, is equivalent to the reciprocal of the generation time in days and equals $\log_{10} \times 3.32$.

TABLE III

GROWTH MEASUREMENTS OF SCENEDESMUS OBLIQUUS DURING CULTURES SIX, SEVEN, EIGHT WITH MICRONUTRIENTS AND CULTURE NINE DEFICIENT IN MICRONUTRIENTS

DAYS AFTER INOCULATION	CULTURE 6 PCV	CULTURE 7 PCV	CULTURE 8 PCV	CULTURE 9 PCV
	ML/100 ML			
0	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00
1.5	0.01	0.00	0.01	0.01
2	0.01	0.01	0.01	0.01
2.5	0.02	0.02	0.01	0.01
3	0.03	0.03	0.01	0.01
3.5	0.04	0.05	0.02	0.01
4	0.06	0.07	0.04	0.01
4.5	0.12	0.11	0.07	0.04
5	0.12	0.12	0.10	0.06
5.5	0.12	0.12	0.11	0.07
6	0.15	0.13	...	...
6.5	0.13	0.13	0.16	0.10
7	0.15	0.16	0.19	0.11
7.5	0.17	0.17	0.22	0.12
8	0.19	...	0.24	0.14
8.5	0.22	0.24	0.30	0.14
9	0.23	0.25	0.30	0.14
9.5	0.24	0.27	0.30	0.14
10	0.28	0.31	0.30	0.14
10.5	0.26	0.31	0.25	0.16
11	0.30	0.36	0.26	0.16
11.5	0.28	0.36	0.26	0.14
12	0.32	0.38	0.30	0.18
12.5	0.40	0.41	0.31	0.16
13	0.40	0.41	0.30	0.15
13.5	0.40	0.40	0.31	0.12
14	0.40	0.45	0.34	0.16
14.5	0.41	0.40	0.34	0.16
15	0.44	0.45	0.37	0.17
15.5	0.49	0.47	0.37	0.17
16	0.52	0.50	0.40	0.18
16.5	0.53	0.53	0.38	0.19
17	0.60	0.55	0.39	0.20
17.5	0.50	0.55	0.50	0.21
18	0.55	0.52	0.65	0.23
18.5	0.50	0.55	0.52	0.21
19	0.52	0.60	0.52	0.21
19.5	0.59	0.60	0.54	0.21
20	0.11	0.60		
20.5	0.15	0.61		
21	0.16	0.13		
21.5	0.17	0.13		
22	0.17	0.15		
22.5	0.23	0.19		
23	0.24	0.22		
23.5	0.28	0.24		
24	0.32	0.29		
24.5	0.30	0.28		
25	0.32	0.28		
25.5	0.32	0.32		
26	0.45	0.45		
26.5	0.50	0.45		

presented in table III. The N analyses from samples taken during culture are given in table IV.

The average N content of the cells from both cultures was similar and did not appear to agree with earlier results regarding increased N removal per cell unit in micronutrient deficient cultures (Krauss, 15). However the regression lines showing the relations between pcv, cell count, and dry weight for the deficient culture 9 and non-deficient cultures 6, 7, and 8 demonstrate that the cells from deficient cultures are much larger and heavier than those in complete media. At a pcv of 0.2 ml/100 ml the weight of the individual cell grown in the complete media is 2.3×10^{-6} mg in contrast to 4.5×10^{-6} mg for deficient cells. This relationship agrees with the different appearance of the algae in the two media. Restricted cytokinesis after karyokinesis in the micronutrient-deficient algae results in giant cells which show only the beginnings of cleavage lines. Though division is prevented, growth continues as evidenced by increase in dry weight and progressive N absorption. Genetically identical, the algae grown in the different media are morphologically and physically unlike and must also differ in their biochemical characteristics. The differences in regression for pcv/dry wt and pcv/cell count for the complete and nutrient-deficient cultures illustrate the errors encountered in accepting these units as identical in cultures grown under a varying physical and chemical environment.

Specific biochemical differences were determined in experiments with two cultures designed to test the validity of the hypothesis for optimum yield and to provide daily samples for inorganic analysis. These cultures, designated 10 and 11, were maintained at a pcv of 0.10 ± 0.03 ml/100 ml by daily harvest of the increment. They were periodically supplied nitric acid in amounts sufficient to replace the nitrate that had been removed. If light, N, and micronutrients were the only limiting factors in earlier cultures, growth in the complete medium should have continued at an undiminished rate. The yield curve in figure 6 demonstrates that this was not the case. The increase in light resulting from removal of cells did increase the yield from 0.046 grams dry weight per liter per day to 0.064 grams in the complete medium and from 0.028 grams to 0.038 grams in the micronutrient deficient medium. Nevertheless, the theoretical yield of 45 grams per day calculated for the complete medium was approached only during the first two days of harvest after which the yield became progressively lower.

TABLE IV

TOTAL NITROGEN IN CELLS OF SCENEDESMUS OBLIQUUS FROM CULTURES EIGHT AND NINE

DAYS AFTER INOCULATION	% TOTAL N (DRY WT. BASIS)	
	CULTURE 8	CULTURE 9
4	7.75	7.16
8	7.23	7.21
14	6.72	6.73
19	6.97	6.75
Average	7.14	6.96

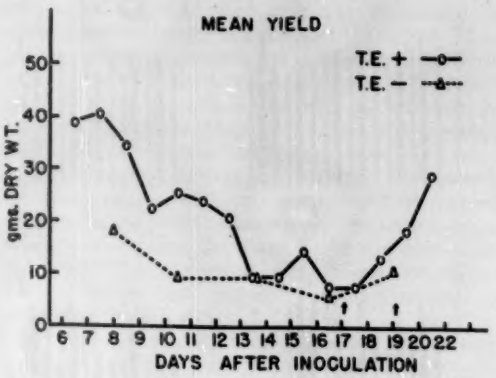


FIG. 6. The mean yield of *Scenedesmus obliquus* calculated for two day periods in culture 10, containing micronutrients (T. E.+), and culture 11, deficient in micronutrients (T. E.–). On the seventeenth day 0.5 ppm of iron was added to each culture and on the nineteenth day one half the initial concentration of micronutrients was added to culture 10.

Reduction in yield could be attributed either to a deterioration of the nutrient supply or to an unknown inhibitor being added to the medium by the algae or bacterial contaminants. Exhaustion of major nutrients from this medium seemed unlikely, but instances of iron precipitation from even fresh media are common in the literature (Myers, 18). Therefore on the seventeenth day 0.5 ppm of iron was added to both cultures. The increased yield during the following two days indicated some beneficial effect. On the nineteenth day the micronutrient complement in culture 10 was supplemented by one half the initial concentration. The increase in yield on the subsequent days was marked. This observation is in agreement with earlier experiments indicating a sharp rise in growth when micronutrients were added. There is a progressive removal of micronutrients from solution in excess of what can be accounted for by strictly metabolic requirements of the algae. Either the precipitation or the complexing of trace elements appears, at least in part, responsible for reduction in yield in older cultures. Whether the complexing of micronutrients could ever limit algal growth in natural waters is open to question. However, clear inhibition of growth under conditions of N sufficiency suggests that the unavailability of micronutrients may be as limiting to growth as the N deficiency suggested by Gerloff et al (10).

The cells harvested from cultures 10 and 11 were analyzed for the major nutrients and calcium. Results of the analyses are given in figure 7. Attention may be directed to quantitative differences between the two cultures and to changes in composition occur-

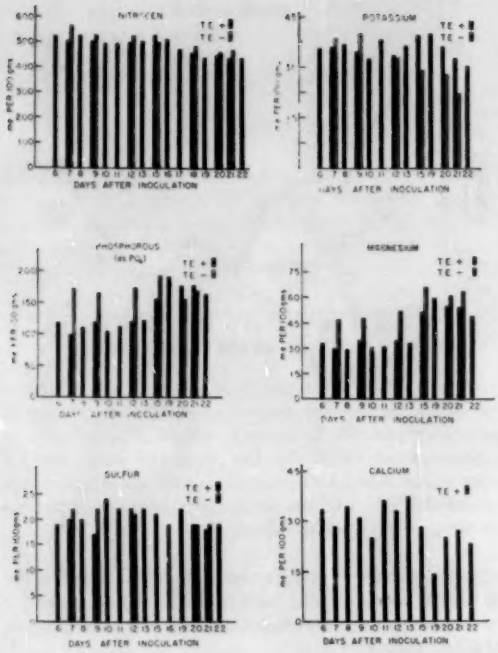


FIG. 7. Inorganic analyses of dried Scenedesmus cells harvested from cultures 10 and 11. Culture 10 (T. E. +) was initially supplied micronutrients. Culture 11 (T. E. −) was deficient in all micronutrients.

ring with time. Of the anions, N and sulfur are found to be relatively constant during the course of growth in both cultures. The finding of a slightly higher N content in the deficient algae is inadequate to establish a statistically significant difference between the two cultures. Phosphorus, however, which was markedly higher during the early period of growth in the deficient culture, is matched by the increased level in the older cells from the complete culture. The tendency toward a wide difference only during the early growth period is also apparent for magnesium. This trend may be interpreted either as the approach to a micronutrient deficient condition even in the culture initially supplied micronutrients or as a secondary phenomenon associated with the longer period in culture of cells growing at a slower rate. The relationship in the case of potassium appears more complex, showing a gradually decreasing content in the cells of the deficient culture and a somewhat more constant level for the cells of the complete culture. The remaining cation, calcium, considered a micronutrient for the algae, is accumulated to some degree in the culture to which it was added. The range in elemental composition for both cultures is summarized in table V. It is now possible to replace the major nutrients on a schedule equivalent to their absorption and, therefore, to maintain the initial nutrient level during the entire course of a culture.

The possibility of loss of nitrogenous compounds to the medium was also investigated. Chitin has been found in the cell walls of the Chlorophyceae and discarded wall material may be broken down to liberate glucoseamine. Watanabe (33) has shown that amino acids may be secreted by blue-green algae and Algeus (1) has demonstrated that *Scenedesmus obliquus* may produce ammonia when supplied organic N. Samples of the medium from culture 10 analyzed for nitrate N and total N indicated that an average of 2.3 % of the N absorbed was returned to the medium in some form other than nitrate. That a significant difference between total N and nitrate N does exist is confirmed by a t-test at the 5 % level. This N fraction has not yet been isolated but may be a by-product of cell division or result from true secretion.

The extent of bacterial contamination was also of major concern. Serious reduction in growth resulting from bacterial contamination has been reported in the literature and cultures were customarily discarded if contamination was detected. However, the demands on equipment and technique needed to maintain a mass culture in a closed, aseptic system favored the alternative approach which would tolerate and if possible control bacteria in an open system. Exploratory studies aimed at finding compounds capable of limiting the growth of bacteria without concomitant injury to the algae were conducted with *p*-aminobenzenesulfonamide. The sulfonilamide was introduced to test tube cultures in concentrations ranging from 0.0001 to 0.02 M. Growth of the algae was halted completely at all concentrations. The cells were destroyed at concentrations above 0.004 M but were still green and somewhat enlarged in concentrations at and below 0.004 M. The controls grew normally. Though this compound could not be used as a selective bacteriostatic the observation that growth is inhibited by an analogue of *p*-aminobenzoic acid strongly suggests the operation of this vitamin in the metabolism of Scenedesmus. The problem of screening for a selective bactericide was deferred but the degree of bacterial contamination was measured during the growth of cultures 4, 5, 8, 9, 10, and 11. Typical of the level

TABLE V

MAXIMUM AND MINIMUM VALUES IN INORGANIC COMPOSITION OF SCENEDESMUS OBLIQUUS FROM CULTURES TEN AND ELEVEN

ELEMENT	CULTURE 10 COMPLETE MEDIUM % (DRY WT. BASIS)		CULTURE 11 MICRONUTRIENT DEFICIENT MEDIUM % (DRY WT. BASIS)	
	Max.	*Min.*	*Max.*	*Min.*
Nitrogen	7.43	6.17	7.74	6.52
Phosphorus	1.82	1.02	1.99	1.60
Sulfur	0.39	0.28	0.36	0.30
Potassium	1.62	1.19	1.53	0.85
Magnesium	0.72	0.36	0.80	0.57
Calcium	0.08	0.03	0.00	0.00

TABLE VI

BACTERIAL POPULATION IN CULTURE TEN CONTAINING MICRONUTRIENTS AND CULTURE ELEVEN DEFICIENT IN MICRONUTRIENTS

DAYS AFTER INOCULATION	BACTERIA PER ML (ON AGAR, 30°C, 48 HOURS)	
	CULTURE 10	CULTURE 11
	In thousands	*In thousands*
0	330	173
1	11,800	2,200
2	31,900	30,000
3	32,600	32,000
5	4,250	4,500
7	6,630	10,000
9	1,550	22,000
11	3,000	23,000
13	700	5,000
15	1,700	11,300
17	1,000	20,000

of contamination is that found in cultures 10 and 11, presented in table VI. Though a number of species were isolated, the bacteria most commonly involved were non-motile, non-spore forming, gram-negative, non-colonial, rod-shaped organisms forming bright orange surface colonies on nutrient agar. These characteristics are common to the genus of saprophytic water bacteria, Flavobacterium. In spite of the high count no direct damage to the algae was observed and the large initial contamination fell to a much lower level after the third day. Inasmuch as the only organic source of carbon, excepting the small amount of citrate introduced with the iron, is the algae it must be concluded that the bacterial population is nourished by cell wall material cast off when aplanospores are liberated and is possibly supplemented by nutrients continuously secreted by the algae as indicated by the presence of non-nitrate N in the medium. An α-naphthol test for free carbohydrates in the medium was negative. The greater number of bacteria in the slowly growing micronutrient-deficient culture suggests that the secretion of metabolites by the cells is more important than cell wall material for nutrition of the bacteria. It is difficult to explain the reduction in bacteria unless some inhibiting substance also reaches an effective level as the culture ages. The possibility of symbiosis cannot be overlooked in evaluating the role of the bacteria in mass cultures, for destruction of the organic waste products of algal growth may be essential to continued productivity. There is little doubt that further work must be done before an understanding of and control of the entire bacterial complex accompanying algae in such cultures can be achieved.

SUMMARY

A new technique is presented for the mass culture of unicellular algae under controlled conditions. Eleven cultures of *Scenedesmus obliquus* (Turp.) Kütz. were grown in 300-liter, polyethylene-lined vats maintained under constant light and temperature in a controlled environmental chamber. A battery of incandescent and fluorescent lamps suspended above the cultures supplied an average illuminance of 1,000 fc and an average irradiance of 0.12 gm cal/cm^2 × sec at the surface of the culture. A stream of 5 % carbon dioxide-in-air served as the carbon source.

1. Growth of *Scenedesmus obliquus* was followed by means of cell count, dry weight, and packed cell volume. Data were presented and regression calculated demonstrating the quantitative relationship between each type of measurement. These relationships were shown to vary markedly under conditions of micronutrient deficiency.

2. Analyses of culture media showed the progressive removal of each of the major nutrient elements. These data served as a guide for the adoption of a culture medium more suitable for prolonged mass culture.

3. A continuous carbon dioxide supply is shown to provide a bicarbonate buffer system by replacing the nitrate anions removed, thereby maintaining an electrostatically balanced system. In the absence of carbon dioxide the bicarbonate is rapidly removed causing a rise in pH to inhibiting levels.

4. Analyses of cells harvested daily established the range of inorganic composition in algae grown in both complete and micronutrient deficient cultures. These data provide a basis for replacement of nutrients absorbed during continuous culture.

5. Applying the principle of the optimum catch a daily harvest of 45 grams dry weight was calculated to be the maximum for each culture. The requisite level of algal population which must be maintained to effect this yield is 7.5×10^6 cells/ml or a dry weight of 150 mg/liter. Under a system of continuous harvest this yield was approached only during the first two days of harvest.

6. Evidence from analyses and growth studies indicates that a major limiting factor in the growth of algae in mass culture is the reduction in availability of micronutrients even though initially introduced into the culture. Supplementary addition of micronutrients permits resumption of rapid growth.

7. The problem of controlling bacterial contamination in an open system is discussed. The initially high bacterial population during the first three days is depressed as the cultures age. Higher populations are found when trace elements are deficient. The use of sulfonilamide as a selective bacteriostatic agent is precluded by its inhibition of growth of Scenedesmus.

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SOME EFFECTS OF DEUTERIUM OXIDE ON THE QUINONE HILL REACTION OF CHLORELLA PYRENOIDOSA CHICK [1,2]

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Since the discovery of the Hill reaction (9, 10, 11, 12), there has accumulated considerable evidence (5, 7, 10, 14, 15, 16, 17, 20) indicating that it is indeed a partial reaction of photosynthesis. And in particular there is evidence that the normally limiting dark reactions of photosynthesis and the Hill reaction are identical (5, 7).

Numerous substances inhibitory to photosynthesis and the Hill reaction have been studied, and in certain cases the site of action of the inhibitor has been localized in more or less detail. The inhibition of photosynthesis of Chlorella by deuterium oxide was shown (6, 18) to have the characteristics of a dark reaction inhibition, but the experimental data give no indication of which dark reaction is sensitive. By means of flashing light experiments, it may be possible to determine whether or not an inhibitor affects the normally rate-limiting dark reaction; thus the inhibition by cyanide was shown (19, 23) not to affect this reaction in Chlorella and Scenedesmus.

Similar studies have not been reported for other inhibitors which inhibit only at high light intensities and the reaction responsible for light saturation of Chlorella photosynthesis (and presumably also of the Hill reaction) remains one for which no specific poison has been certainly established. The experiments reported here were preliminary to an attempt to decide whether deuterium oxide is an inhibitor of this reaction. No decision can be made on the basis of data so far obtained. The present paper is particularly concerned with a comparison of the effects of deuterium oxide on the light saturation curves of photosynthesis in Chlorella and of the quinone Hill reaction of whole Chlorella cells. As an aid in interpreting these results, the effect of pH on the rate of the quinone Hill reaction of Chlorella at low and at saturating light intensities is also reported.

MATERIALS AND METHODS

The strain of *Chlorella pyrenoidosa* employed was obtained originally from Dr. Robert Emerson. It was grown autotrophically in bacteria-free medium of the following composition:

H_2O	1000	ml
KNO_3	2.5	gm
KH_2PO_4	2.5	gm
$MgSO_4 \cdot 7\,H_2O$	5.0	gm
$Fe_2(SO_4)_3 \cdot 6\,H_2O$	0.018	gm
trace elements: B, Mn, Zn, Cu, Mo, Co.		

Loop inoculated cultures were incubated at 28°C with a continuous stream of 5 percent carbon dioxide in air bubbling through 200-ml cultures. The culture flasks were illuminated from below by a bank of white fluorescent lights with an incident intensity of about 100 fc.

The cultures were removed from the culture chamber during their logarithmic growth phase, and left for at least 12 hours in the dark at 2°C before use, and all operations on the cells, preliminary to setting them up in an experimental run, were performed at 2°C. Cells were centrifuged out of the culture medium (pH 4.8) and resuspended in culture medium to the desired cell density. Cell density was measured by obtaining the optical density, at 675 mμ, of a suitable dilution of suspension in a 1 cm thick cuvette with a Beckman DU spectrophotometer; optical densities of suspensions at one half their experimental concentrations varied from 0.8 to 1.2. In experiments lasting several days the stock cell suspension was stored in the dark at 2°C.

In order to insure that comparable salt solutions in D_2O or H_2O had exactly the same content of dissolved substance, suitably small aliquots of a stock solution were dried down in a vacuum desiccator and made up to their former volume in D_2O or H_2O, as the case might be. In the case of Warburg No. 9 carbon dioxide buffer (21), it was found that a solution subject to the treatment described had a pH only negligibly different from the stock solution from which it was derived and, therefore, only a negligible amount of CO_2 was removed from it.

Cell suspensions in a desired medium were obtained from the stock suspension in culture medium by sedimenting the cells by centrifugation from a suitable aliquot of stock, discarding the supernatant, wiping away as much of the supernatant adhering to the tube as possible, resuspending the cells in the desired medium, and diluting to the same volume as before. Cells to be used in Warburg No. 9 buffer were washed in this buffer once between the first centrifugation and resuspension in the final experimental solution.

Cells to be used in a Hill reaction with quinone could not be used immediately after having the culture medium replaced by a medium of higher pH, or at least this was true for media in the higher pH

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[3] Part of the work described here was done while the author was the recipient of the Conway MacMillan Fellowship of the University of Minnesota. Present address: Institute of Radiobiology and Biophysics (Fels Fund), University of Chicago, Chicago 37, Illinois.

ranges studied, for there was a period of adaptation in the new medium before the cells were stable in time with respect to their ability to do the quinone Hill reaction. Thus the rates of the quinone Hill reaction obtained at different times after replacing culture medium with M/15 potassium phosphate buffer of pH 7.47 and allowing the cell suspension to stand in the dark, are:

40	minutes	.....	0.35	mm of	Brodie/minute	
205	"	.....	0.55	"	"	"	"
587	"	.....	0.61	"	"	"	"
1377	"	.....	0.54	"	"	"	"

Because of this phenomenon, cells were always adapted to a medium other than culture medium for at least three hours and not more than twelve.

Quinone solutions were prepared from sublimed quinone which was dissolved in 0.01 *N* H_2SO_4.

The D_2O was used without purification as supplied by the Stuart Oxygen Company by authorization of the U. S. Atomic Energy Commission. The nominal concentration of the D_2O was specified as 99.8 percent. Isotope analyses with a mass spectrometer on diluted aliquots of this heavy water were performed with a precision sufficient to confirm the first two digits only.

The light source was a 500-watt reflector spot tungsten filament bulb. An acid solution of ferrous sulfate, which was shown to exclude heat effects in the manometric measurements, was interposed between the light source and the experimental material. Changes in light intensity on the experimental material were effected, down to 14 percent of full light, with calibrated screens; lower light intensities were obtained by cutting down the voltage across the light source and measuring these lower intensities, relatively, with a General Electric exposure meter.

Rate measurements of Hill reactions or photosynthesis were usually made using Warburg manometric technique, with vessel constants of from 0.88 to 0.91 for oxygen at 14°C and with 2.2 ml of liquid in the vessel. Unless otherwise specified the Warburg vessel was filled in dim light with 2 ml of a suitable cell suspension which had just previously been taken from darkness at 2°C. The vessel had no side arm or center well and no alkali was used. Two tenths ml of quinone solution was inserted in the gas cock capillary, and, after putting the vessel in the darkened constant temperature bath, the solution was flushed in with hydrogen which had passed through a "Deoxo" catalytic deoxygenation cartridge; the vessel was thoroughly flushed out with this hydrogen. The quinone was in contact with the cells for from 17 to 42 minutes before the light was turned on. When light intensity was a critical factor to control, all runs in a series were made at the same position in the bath, using the same vessel and the same light source run at a known and constant voltage.

Unless otherwise specified, the concentration of quinone in an experimental cell suspension before the light was turned on varied from 0.020 to 0.022 percent. The cell suspension density varied from 3.5 to 5.0 μl of wet packed cells per ml, and therefore respiration should have been inhibited by the quinone to a rate below the limit of manometric measurement (5). That photosynthesis is absent or negligible under these conditions was demonstrated in an experiment in which the mass spectrometer was employed as a measuring instrument. The general method has been described previously (4) except for a change in the spectrometer gas inlet leak which was incorporated into the male joint of the Warburg-type vessel as explained elsewhere (3). The measurements, although made by a different physical method, were entirely comparable to manometric measurements except that both oxygen and carbon dioxide partial pressure charges were determined independently by the mass spectrometer. The results of the experiment are shown in figure 1. During the light period only a negligible change in carbon dioxide was observed.

In these experiments there were frequent cases where a relatively high initial rate of oxygen production from quinone fell, within 10 to 20 minutes, to a lower rate which remained quite constant for some time. There was often some difficulty, therefore, in determining an initial rate, even though 2-minute readings were taken at the beginning of a light period. Figure 2 illustrates the phenomenon being discussed.

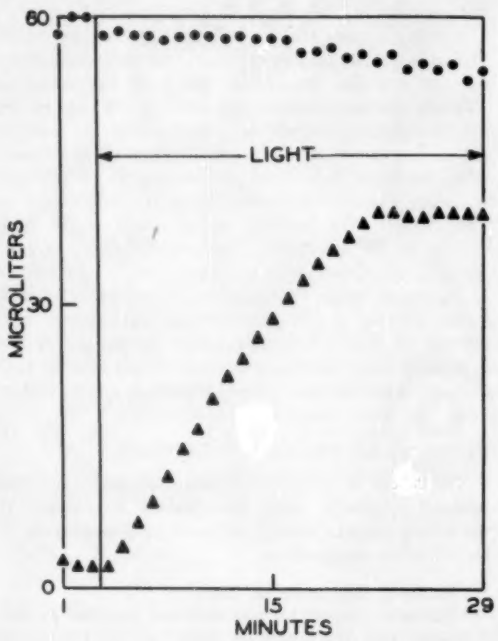

FIG. 1. Time course of oxygen and carbon dioxide changes during the quinone Hill reaction with whole Chlorella cells. Ordinate: volume of oxygen (triangles) or of carbon dioxide (solid circles) present in the vessel under standard conditions. Data were obtained using a recording mass spectrometer. Cells suspended in mannitol borate buffer, pH 7.35. Temperature, 20.6°C.

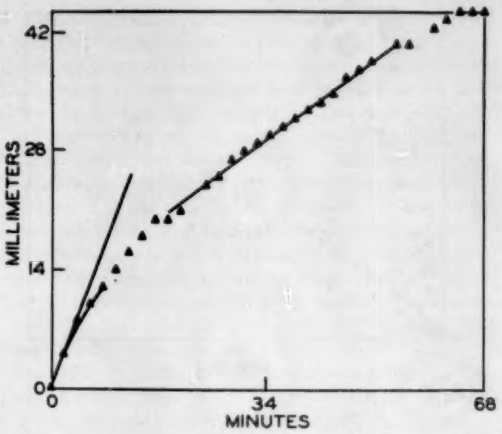

FIG. 2. Illustration of the difference between "initial" and "linear" rates of the quinone Hill reaction of Chlorella. Ordinate: pressure change in mm Brodie solution. Illumination began at zero time. Data were obtained manometrically. Cells suspended in phosphate buffer, pH 7.47. Temperature 14.4°C. Lines are drawn with slopes equal to "initial" and "linear" rates as discussed in the text.

The most nearly reproducible rate measurements could be made on the "linear" part of the curve, but in order not to completely lose sight of initial rates, which are suitable for a simpler kinetic interpretation, they were estimated in the following way: a smooth curve was drawn through the points and the amount of gas produced in the light corresponding to the point where this curve crossed the 6-minute ordinate, was taken as a measure of initial rate.

RESULTS AND DISCUSSION

THE EFFECT OF D_2O ON THE SATURATION CURVES OF THE HILL REACTION AND PHOTOSYNTHESIS: As is shown in figure 3, the results of Craig and Trelease (6), indicating that D_2O inhibits a dark reaction in the photosynthesis of Chlorella, could be reproduced with the material used in these experiments. The effect of D_2O on the quinone Hill reaction, however, is quite different as can be seen from figure 4, where the upper set of saturation curves was obtained at pH 6.6 and the lower set was obtained at pH 7.43. In gathering the data at pH 6.6 no systematic attempt was made to adapt the cells to the medium of pH 6.6 for at least three hours. However, this seems not to have affected the validity of the results, judging from the internal consistency of the data and from the ratio of the saturation rate at this pH to that of the lower set of curves at pH 7.43, which is just about the ratio one would expect on the basis of data obtained relating pH and the saturation rate of the quinone Hill reaction. The character of typical data used in getting the points of figure 4 is shown in figure 5. Unlike photosynthesis, where the inhibition by D_2O markedly decreases at low light intensities, there appears to be no diminution of the inhibition of the quinone Hill reaction at low light intensities.

In order to interpret these results, it is necessary to consider the relative effects of D_2O and H_2O solutions, with identical salt composition, on the degree of dissociation of weak acids, with particular reference to weak acids of critical physiological importance (e.g. enzymes) within the cell. It is known that pH markedly influences the Hill reaction velocity of isolated chloroplasts (2, 14, 15) and data obtained in this laboratory show that there is a sharp maximum of the "linear" rate of the quinone Hill reaction in H_2O media at about pH 7.4 in both M/15 potassium phosphate buffer and in mannitol borate buffer.[4] This effect is presumably due to changes in degree of

[4] This consisted of M/10 boric acid adjusted to the desired pH with NaOH, 7.18 gm of KCl per liter, and 8 gm of mannitol per liter.

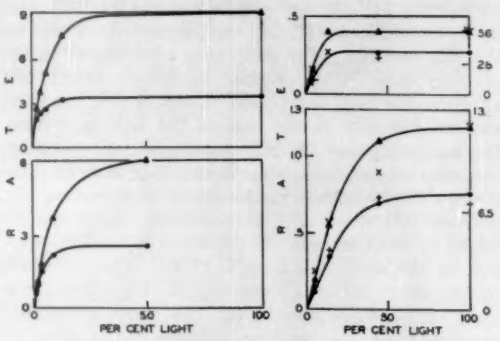

FIG. 3 (*left*). The effect of D_2O on Chlorella photosynthesis. Ordinates: photosynthetic rate (units arbitrary). Abscissa: incident light intensity as percent of maximal intensity. Lower graph drawn from representative data of Craig and Trelease (6) who used *Chlorella vulgaris* at 23°C in Warburg No. 9 buffer. Upper graph drawn from the author's data on *Chlorella pyrenoidosa* in Warburg No. 9 buffer at 13.7°C. The triangles are for photosynthesis in H_2O, and the solid circles are for photosynthesis in D_2O.

FIG. 4 (*right*). Saturation curves of the quinone Hill reaction of Chlorella in D_2O and H_2O. Abscissa: incident light intensity as percent of maximum. Left hand ordinate is "linear" rate of reaction in mm Brodie per minute. Right hand ordinate is "initial" rate in mm Brodie increase during first 6 minutes. The triangles are for "linear" rate in H_2O and the closed circles are for "linear" rate in D_2O. The curves are drawn through the points determined from linear rate. The X's are for "initial" rate in H_2O and the plus signs are for "initial" rate in D_2O.

Upper set of curves: Determined at pH 6.6 in potassium phosphate buffer. Concentration of quinone present before light was turned on was 0.011 percent. Temperature 14.0°C.

Lower set of curves: Determined at pH 7.43 in mannitol borate buffer. Temperature 14.6°C.

dissociation of weak acids of critical physiological importance accompanying changes in pH. Is it not possible that D_2O exerts its influence on the Hill reaction also by affecting the degree of dissociation of weak acids?

Rather than rely on ambiguous theoretical considerations, it was felt that an empirical approach to this problem would be more satisfactory. It consisted in determining how "pH inhibition" affects the saturation curve of the quinone Hill reaction in an H_2O medium. If it has the characteristics of a dark reaction inhibition, then it is not possible to attribute the D_2O inhibition of the quinone Hill reaction simply to the effect of D_2O on degrees of dissociation of weak acids. A comparison made between the saturation curves at pH 6.6 and at pH 7.3 showed that "pH inhibition" disappeared at low light intensities.

The inhibitory behavior of D_2O on the quinone Hill reaction appears to be like that shown by narcotics (17, 21, 22), by ultraviolet light (1, 13), and by hydroxylamine (17, 23) on photosynthesis and on the Hill reaction. The difficulties encountered in explaining these latter peculiar inhibitions would also apply to the D_2O inhibition. There is yet, however, another difficulty in the case of the D_2O inhibition, for, supposing that the Hill reaction is a partial reaction of photosynthesis, why should D_2O have qualitatively different effects on photosynthesis and on the quinone Hill reaction? This difficulty might be resolved by looking upon the inhibitors in the Hill reaction as the combination of D_2O and quinone which has an effect different from that of deuterium oxide alone on photosynthesis. And this could be related to observations of previous workers (5, 7) that, at light intensities insufficient to saturate the quinone Hill reaction, its rate is considerably lower than the rate of photosynthesis. It follows from these considerations that the inhibition of Hill reactions by D_2O, using oxidants other than quinone, may not differ qualitatively from the inhibition of photosynthesis.

THE QUINONE HILL REACTION OF CHLORELLA FROZEN IN DRY ICE: In an attempt to obtain material which would be more stable in time than fresh Chlorella kept in the dark at 2°C, a Chlorella suspension with an optical density at 675 mμ of 1.20 (when at one half its experimental concentration), which had been stored for about 40 hours in the dark at 2°C in M/15 potassium phosphate buffer of pH 7.3 and then made up as small aliquots in H_2O or D_2O solutions of this same salt composition, was frozen down quickly in dry ice. By analogy with results on isolated chloroplasts (8) which had been similarly treated, the Hill reaction activity of these cells should remain constant for at least several months, and, by spot runs in saturating light of the quinone Hill reaction of cells frozen in H_2O, it was shown that it remained constant at least during the time when cells frozen in H_2O and D_2O were being compared.

An attempt to determine comparative saturation curves of the quinone Hill reaction of cells frozen in H_2O and D_2O did not result in data which were suitable for a simple kinetic interpretation, and are therefore not plotted all together as a saturation curve. Instead, examples of the kind of data obtained at saturating light intensity and at much lower intensity are shown in figure 6. The upper series of D_2O points—the triangles being for reaction in H_2O and the closed circles for reaction in D_2O—illustrates the peculiar, continuously bending character of the D_2O curve here as compared with the D_2O curve of figure

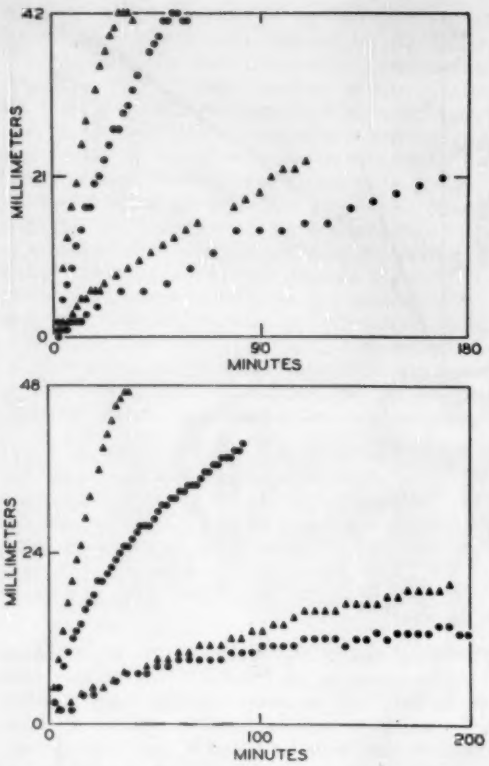

FIG. 5 (*upper*). Data representative of the results upon which figure 4 was based. Ordinate: gas production in mm Brodie solution. Illumination began at zero time. Reaction in H_2O, triangles; reaction in D_2O, solid circles. Upper two series of points (triangles and solid circles) obtained at a saturating (100%) light intensity. Lower two series of points obtained at 4.9% light intensity. Cells suspended in mannitol borate buffer, pH 7.45. Temperature 14.6°C.

FIG. 6 (*lower*). Time course of the quinone Hill reaction in H_2O and D_2O with previously frozen Chlorella. Ordinate: gas production in mm Brodie solution. Reaction in H_2O, triangles; reaction in D_2O, solid circles. Illumination began at zero time. Upper two series of points (triangles and solid circles) obtained at a saturating (100%) light intensity. Lower two series of points obtained at 1.1% light intensity. Cells suspended in phosphate buffer, pH 7.3. Temperature 13.8°C.

5, where saturating light was also used, but with fresh Chlorella. At low light intensity the D_2O curve again has a continuously bending character, and, unlike the D_2O curve of the quinone Hill reaction with fresh Chlorella at low light intensities (see figure 5), the D_2O curve here, as far as one can tell, corresponds initially with the H_2O curve. These data, then, demonstrate two differences between the quinone Hill reaction of fresh and frozen Chlorella: 1) the stability of the reaction of frozen Chlorella during the time the reaction runs is less than that of fresh Chlorella, especially when comparison is made in D_2O; 2) at low light intensities no difference in initial rate of reaction in D_2O and in H_2O could be detected with frozen cells, although differences are evident with fresh cells; at high light intensities the percent inhibition of initial rate by D_2O in frozen cells is much like the percent inhibition in fresh cells.

Summary

1. The effect of deuterium oxide on the quinone Hill reaction of whole *Chlorella pyrenoidosa* cells is qualitatively different from its effects on photosynthesis.

2. Chlorella cells which had previously been frozen in dry ice were capable of a quinone Hill reaction but there were certain kinetic differences between this reaction in previously frozen and in unfrozen cells, and the effect of D_2O was somewhat different in the two cases.

The author wishes to thank Dr. A. H. Brown for suggesting the problem to him, and for helpful advice and discussion throughout the course of the work.

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THE INTERRELATION BETWEEN CO_2 METABOLISM AND PHOTOPERIODISM IN KALANCHOË[1]

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Many studies have been made of the flowering response of plants exposed to alternating conditions of light and darkness. The present state of knowledge on this subject is reviewed by Lang (12). These studies have given rise to a number of schemes which attempt to explain photoperiodism by means of specific reactions which are supposed to occur in the dark and light phases of photoperiodic cycles, and by the formation in the leaf of flower-promoting and/or inhibiting substances under the influence of light and dark reactions. Diurnal endogenous rhythms of alternating "photophil" and "skotophil" phases have also been invoked to account for the varying behavior of long and short day plants (4), but no direct evidence of diurnal changes in metabolism has so far been presented to substantiate this theory.

In contrast with this wealth of speculation little has been done to investigate changes in the metabolism of plants, resulting from alterations in the photoperiodic regimes to which the plants are submitted. In spite of the well-established fact that a supply of CO_2 during the light phase is essential for the flowering response of short day plants (soybean: Parker and Borthwick, 13; Kalanchoë: Harder and von Witsch, 11) the change in assimilation rate during induction has not been directly investigated, nor has the need for CO_2 during the dark phase of the cycle.

Some interesting experiments have been carried out on isolated leaves and shoots taken from plants exposed to different day-lengths (Bode, 2; Schmitz, 15). These data, which will be taken up in more detail later, apparently show that, in Kalanchoë plants, the length of day affects both the photosynthetic rate in the light and the pattern of CO_2 production in the dark.

The suggestive nature of these findings, and particularly the general absence of basic information in this field, indicated the need for a more thorough investigation of assimilatory and respiratory changes in relation to photoperiodism. Such an investigation, it was felt, must of necessity be carried out with whole plants, at least at first, so that correlations between metabolic behavior and flowering could be made where possible.

It is well known that the group of plants known as succulents have diurnal fluctuations in their organic acid level and that they have the ability to fix large amounts of carbon dioxide in the dark (1, 3, 5, 18, 19, 20). With these facts in mind, it was decided to study the CO_2 metabolism of a succulent plant as a function of photoperiod.

The plant selected for study was *Kalanchoë Blossfeldiana*, a short-day plant, whose compactness, ease of handling, and slow growth rate make it well suited to the present purpose. It had already been used for investigations both of flowering (Harder, 9) and of leaf metabolism (2, 15). The only disadvantage of Kalanchoë is the large number of short days needed for floral induction. Under the conditions of temperature and light used, a minimum of 13 or 14 short days are required for any flower primordia to be subsequently formed, while a maximum response is only obtained with 20 to 25 short days. This disadvantage has so far not proved serious.

MATERIALS AND METHODS

Kalanchoë Blossfeldiana var. Tom Thumb was used for most of the experiments described, but in the preliminary experiments at Imperial College, London, another dwarf variety, Ernst Theide, was employed. The plants at Harvard were raised from cuttings taken from plants which had been maintained on long days. They were then grown in air-conditioned light-rooms where the temperature was maintained at 19°C and the relative humidity at 75 %. The length of day was maintained at 16 hours. A light intensity of 1500 fc at the level of the plants was obtained by using incandescent and fluorescent lamps, balanced in such a way as to produce a spectral distribution of light intensity similar to that of sunlight.

For most of these experiments, the plants were enclosed in gas-tight chambers as shown in figure 1. The seal is effected by affixing a slit piece of 7/16" (O.D.) gum rubber tubing around the lower portion of the stem by filling in the space with latex. The tubing is slightly larger than the hole in the brass base plate of the chamber. The base plate is assembled and when bolted together forms a gas-tight seal around the rubber tubing enclosing the stem. The Lucite cylinder slips over the plant, and is pressed against a rubber gasket in the base plate by another brass plate at the upper end. Air is circulated by means of a small inlet nozzle in the base plate and an outlet in the top of the Lucite cylinder. The orifice of the inlet is only 0.5 mm in diameter, and this causes the air to enter in a jet, thereby facilitating mixing. When tested, the chamber did not leak even when subjected to 14 to 20 lbs pressure per square inch, which is many times that used. This type of chamber has proven to be very effective, especially since long-term experiments with the same plant are possible.

The use of a gas-tight chamber, though essential

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[3] The contents of this paper constitute a part of a thesis submitted by I. Spear to the Graduate School of Harvard University in partial fulfillment of the requirements for the Ph.D. degree, May 1953. Present address: Department of Botany, University of Texas, Austin, Texas.

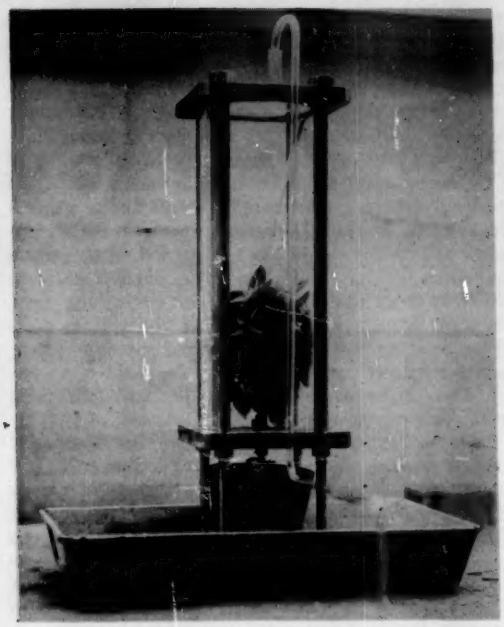

FIG. 1. Gas-tight plant chamber used to study CO_2-metabolism of *Kalanchoë Blossfeldiana*. Description in text.

for metabolic experiments, has the disadvantage that the temperature within the chamber varies with the lighting. Although the room was maintained at 19°C the temperature within the chamber reached 29°C (by thermometer) under the lights. Although the leaves were doubtless cooled somewhat by transpiration in the rapid current of air, still their temperature was certainly not constant.

Most of the CO_2 measurements were made by means of an Infra Red Gas Analyzer (supplied by Messrs. Sir Howard Grubb, Parsons and Company), which gave instantaneous direct readings of the CO_2 content of the air entering and leaving the chambers. Because the CO_2 content of the incoming air was not completely constant, the data are presented as a percentage of the CO_2 content of the incoming air. The absolute values for CO_2 content varied between 0.04 and 0.05 %. For the preliminary experiments NaOH absorption towers were used in addition. For this purpose and for the regulation of flow rates in all of the experiments, a modification of the apparatus described by Porter, Pal and Martin (14) was used. A rate of flow of 27.5 ± 0.5 liters per hour was maintained through the chamber during the 24 or more hours of a run. A detailed description of the modified apparatus, and of the construction of the chambers, is given by Spear (17).

RESULTS

PRELIMINARY EXPERIMENTS: The first experiments were performed at Imperial College, London, in the greenhouse, with the plants therefore subject to varying conditions of temperature and light. Cuttings with their bases in diluted Hoagland's solution were used. The total CO_2 exchange during a period of several hours in the light or in darkness was determined by absorption in towers containing NaOH. The results of one series are summarized in table I. Although the data show some variability, particularly in the light phase, certain general conclusions are evident. In all but one case, there is a net uptake of CO_2 in the dark, indicating that the rate of fixation exceeds that of respiration. For the plants on short day it can be seen that, as the number of short days of pretreatment increases, the uptake of CO_2 in the dark increases, reaching very considerable values. Correspondingly, the net uptake of CO_2 in the light (photosynthesis) decreases. The plants on long day show no such trend, but only minor variations. It is evident, therefore, that the total CO_2 metabolism is strongly influenced by photoperiodic treatment.

Other preliminary experiments made it clear that closer control of external conditions was essential, and that whole plants rather than cuttings would be desirable. All of the succeeding experiments were therefore performed in the air-conditioned light rooms at Harvard, using whole plants. The plants were set at a fixed distance from the lights, with the entire shoot enclosed in the chambers shown in figure 1.

THE DIURNAL VARIATION OF CO_2 METABOLISM: Plants which had always been maintained on non-flower-inducing 16-hour photoperiods ("long days") were placed in the chambers and their CO_2 exchange was followed for one or two 24-hour cycles with long days. They were then transferred to 8-hour photoperiods ("short days"), and were at intervals replaced in the chambers for 24 hours or more, in order to study their gas exchange under short day conditions after having received increasing numbers of short days.

Figures 2 and 3 present the data from a single plant so treated. For ease of presentation the dark and light phases are presented separately, figure 2 giving the CO_2 metabolism in the dark phase. The broken lines show the CO_2 uptake during the 8-hour dark periods following exposure to 16 hours light. Two successive "nights" are shown for the plant, which had not previously received any short days. When the lights go off at midnight (24 hrs.) photosynthesis ceases but there remains a small, though

TABLE I

UPTAKE OF CARBON DIOXIDE BY KALANCHOË CUTTINGS ON LONG AND SHORT DAYS

NUMBER OF SHORT DAYS	CO_2 UPTAKE IN MG CO_2/HR × DM^2			
	EXPERIMENTAL PLANTS ON SHORT DAY		CONTROL PLANTS ON LONG DAY	
	Dark	*Light*	*Dark*	*Light*
13	0.05	1.27	0.03	0.55
22	0.44	0.92	...	0.80
28	0.47	0.87	−0.03	0.70

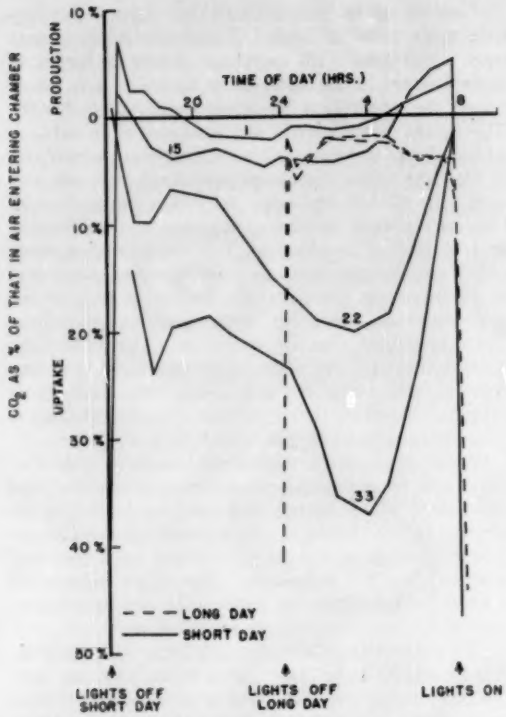

FIG. 2. Carbon dioxide metabolism of Kalanchoë in dark phase under long-day conditions (broken lines) and during the 1st, 15th, 22nd, and 33rd long nights (solid lines).

significant, net dark uptake of CO_2. When the lights come on at 8:00 A.M. photosynthesis resumes and there is a precipitous increase in the rate of uptake of CO_2. This behavior is as would be expected for a succulent plant.

The solid lines show the corresponding behavior in the 16-hour dark phases following 8-hour photoperiods. In the first of these long nights (topmost curve of fig 2) the net CO_2 exchange is small, but in the 15th, 22nd, and 33rd long nights there is a steady increase in the rate of dark fixation of CO_2. The fixation passes through a characteristic cycle, increasing to reach a maximum not later than 12 hours after the onset of darkness, and then decreasing to reach values close to zero before the lights come on at 8:00 A.M.

Figure 3 shows the course of CO_2 metabolism during the light phase. Again the broken lines represent the CO_2 uptake under long day conditions, before the plant has been exposed to short days. In the two experiments recorded, photosynthesis begins rapidly and after a slight recession shows a steady tendency to increase throughout the light period, falling very abruptly as soon as the light is turned off. The solid lines show that the course of CO_2 exchange after exposure to short day cycles is very different. Already after one short day there is a temporary fall in the rate of uptake, followed by a steady rise throughout the rest of the light period. In the other curves these changes become progressively more marked. After 14 cycles of short days the preliminary fall in photosynthesis brings the uptake almost to zero in 2–4 hours; after 21 short days the uptake after 2 hours gives place to a loss of CO_2, while after 32 cycles loss of CO_2 begins soon after the light is turned on, again passing through a maximum. The maximum point is followed in all cases by a rapid increase in CO_2 uptake, and by the end of the light period all the curves approach the same maximum value, which is not very different from that of the plants kept in long days. In other words, plants which have received 33 short days show a net loss of CO_2 during the light phase, and a large net uptake of CO_2 in the dark.

These experiments have been repeated four times with similar results.

INTERRUPTION OF DARK PERIOD: In "short day plants," that is, plants which initiate flowers only after one or more long nights, the effectiveness of a long dark period may be completely annulled by a short period of illumination (8), particularly when given near the mid-point of the dark period (10). It was therefore important to determine whether a brief interruption of the dark period affected the metabolic changes described above.

In the first experiment of this sort, a plant was studied under 16-hour long days as before, and then transferred to 8-hour short days. After six such short days it was replaced in the chamber and found to have a CO_2 metabolism characteristic of plants ex-

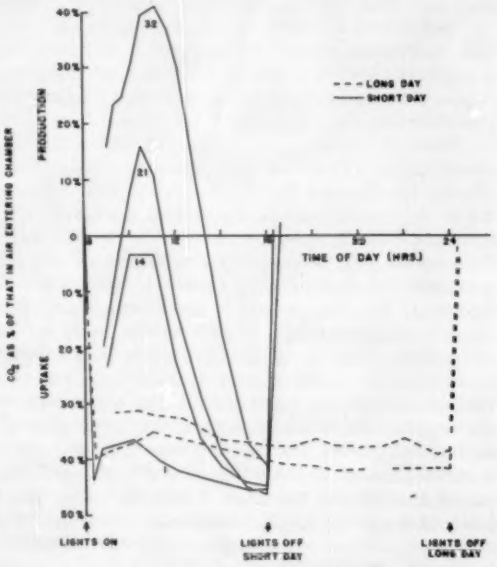

FIG. 3. Carbon dioxide metabolism of Kalanchoë in light phase under long-day conditions (broken lines) and after 1, 14, 21, and 32 long nights (solid lines). Same plants as in figure 2.

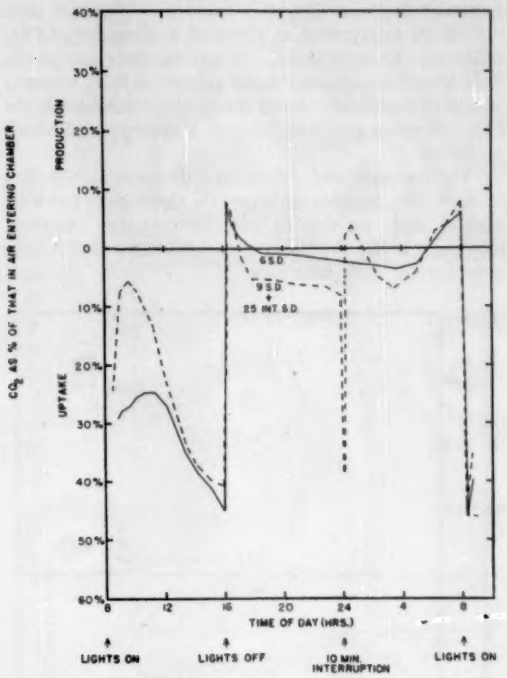

FIG. 4. The diurnal variation in CO_2 metabolism of Kalanchoë after 6 short-day cycles (solid line), and after 9 short-day cycles with 25 additional interrupted short days (broken line). The 10-minute interruption with 1500 fc was at midnight (24 hrs.), the mid-point of the dark period.

posed to a few short-day cycles (i.e., between those shown for 1 and 14 days in figures 2 and 3). The data are shown in figure 4 (solid line). After nine short days the 16-hour dark period was interrupted at its mid-point by 10 minutes of full light (1500 fc). This treatment was continued daily for an additional 25 days, and the CO_2 metabolism of the plant was re-examined at intervals. It was found that the interruption markedly decreased the further development of the characteristic short day pattern, so that there was relatively little increase in the dark uptake of CO_2, and no net production of CO_2 in the light (fig 4, broken line, cf. fig 2 and 3). It appears that interrupting the long dark period, after 9 unbroken short days, is not completely effective in arresting the metabolic changes, but does so to a considerable extent. However, this plant when returned to long days later produced two single flower primordia, even though under the conditions of these experiments no other plants have ever formed flowers with a treatment of less than 13 uninterrupted short days.

In subsequent experiments no pretreatment with short days was given, but plants grown in long days were exposed for prolonged periods to cycles of 8 hours light and 16 hours darkness, with and without interruption of the dark period. The data from one of these experiments is presented in figures 5 and 6. Here again, both plants were first studied under long days, of which two such cycles are averaged and presented in figure 5A. The plants were then transferred to 8-hour photoperiods and their carbon dioxide metabolism was re-examined during the 1st, 7th, 14th, 21st, and 35th cycle (figs 5B through 6D respectively). The broken lines in figures 5 and 6 refer to the plant that received interrupted long nights, while the solid lines refer to the uninterrupted control. The interruption comprised 10 minutes of full light intensity. Although the plants were selected for uniformity their leaf areas were not identical, which accounts for the small discrepancy in the replicates in figure 5A.

It is evident that the plant grown in uninterrupted long nights developed a progressively increasing dark CO_2 uptake and an associated transient production of CO_2 in light, quite similar to those shown in figures 2 and 3. In the companion plant, however, which received briefly interrupted dark periods, this progressive fixation and production of CO_2 is almost completely absent. It is to be noted that the total duration of its light and dark periods does not differ appreciably from that of the controls. It follows that the metabolic changes resulting from short days are true photoperiodic phenomena and are not the result of differences in total light received.

EFFECT OF PROLONGED DARK AND PROLONGED LIGHT PERIODS: It is clear from figures 2, 6C and 6D that the CO_2 fixation in darkness decreases rapidly after the first 12 hours and has usually ceased before the end of the dark period. A study was therefore made of the CO_2 relations in prolonged darkness. Figure 7 shows the effect of such a prolonged dark period on a plant which had previously received 34 short day cycles. During the first 12 hours of dark the typical increase in dark fixation of CO_2 occurs, followed by a rapid decline, so that after 16 hours (i.e., at 8:00 A.M.), dark uptake gives place to continuous production of CO_2. Presumably this is the normal rate of respiration for this plant. The slow decrease in the rate of CO_2 production during the remainder of the 48 hours of darkness may be explained by a decreasing supply of substrate. It is interesting and significant that the 15- to 16-hour period which seems to be required for the dark fixation to run its course corresponds exactly to the optimal length of the dark period, as determined by Harder (9), on the basis of numbers of flowers formed. This observation may provide a physiological meaning for such an optimum. It will be noted also that there is no sign of an endogenous rhythm in the dark CO_2 fixation, nor in the normal respiration rate.

When the lights were turned on at the end of this 48-hour dark period the burst of CO_2 production was similar to that noted after a 16-hour dark period. This means that the substrate responsible for this burst of CO_2 has not been decomposed in prolonged darkness.

The effect of prolonging the light period to 24

hours on plants pretreated with 34 short-day cycles is shown in figure 8. Following upon the usual immediate burst of CO_2 production, which is complete in 4 hours or so, photosynthesis rapidly increases and then remains constant. Again there is no sign of an endogenous rhythm.

Discussion

1. The Fixation of CO_2 in Dark: In plants pretreated with short-day cycles, CO_2 uptake ceases immediately on darkening and then begins again at an increasing rate, reaching a maximum velocity in about 12 hours. The plants kept in long days show only a very slow uptake of CO_2 in darkness. The most probable interpretation of this phenomenon is that an enzyme system which catalyzes the dark uptake of CO_2 is produced during the long dark period. The properties which this enzyme must have are discussed further below.

In so far as they are comparable these data agree with those of Schmitz (15), who found that when leafy shoots from plants receiving long nights of 12 or 18 hours' duration were placed in the dark in CO_2-free air, they evolved very little CO_2 (at 20°C) for the first part of the dark period, but that after 12 hours of darkness CO_2 was evolved. Schmitz's data can all be interpreted in terms of a retention of CO_2 following the light phase. When the duration of the light phase was decreased the amount of CO_2 retained was also decreased. Even under these conditions the CO_2 evolution generally showed a minimum at about 12 hours.

The increase and subsequent decrease in the rate of dark CO_2 fixation in plants on short days (or long nights) may be due to two factors: (a) stomatal movements, (b) the production and removal of a CO_2 acceptor by dark reactions.

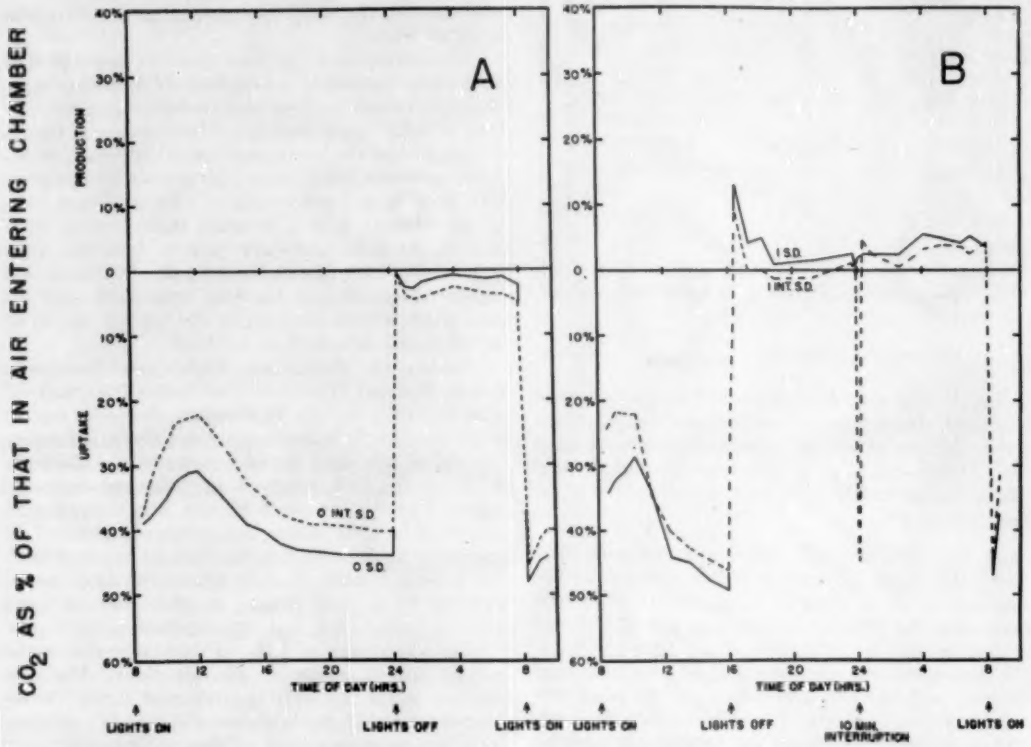

Fig. 5. The diurnal variation in CO_2 metabolism of Kalanchoë under long-day conditions (A) and after one long night (B) with (dashed line) and without (solid line) 10-minute interruption of the long dark period by 1500 fc. In A the dashed line represents the metabolism of the plant which will receive the interrupted nights.

(a) Schwabe (16) has shown in the Chrysanthemum that stomatal behavior in the dark period depends upon the previous photoperiodic regime. Plants given short days show a rapid closure at first, followed by opening in darkness, so that by the end of a 16-hour dark period the aperture is not much less than the maximum. Plants given long days show immediate stomatal closure and no appreciable opening in the dark. Schwabe has qualitatively observed similar behavior in Kalanchoë. If this behavior were to apply to the plants in our experiments it would mean that in the plants given long days external CO_2 would be largely excluded, and it might help to explain the

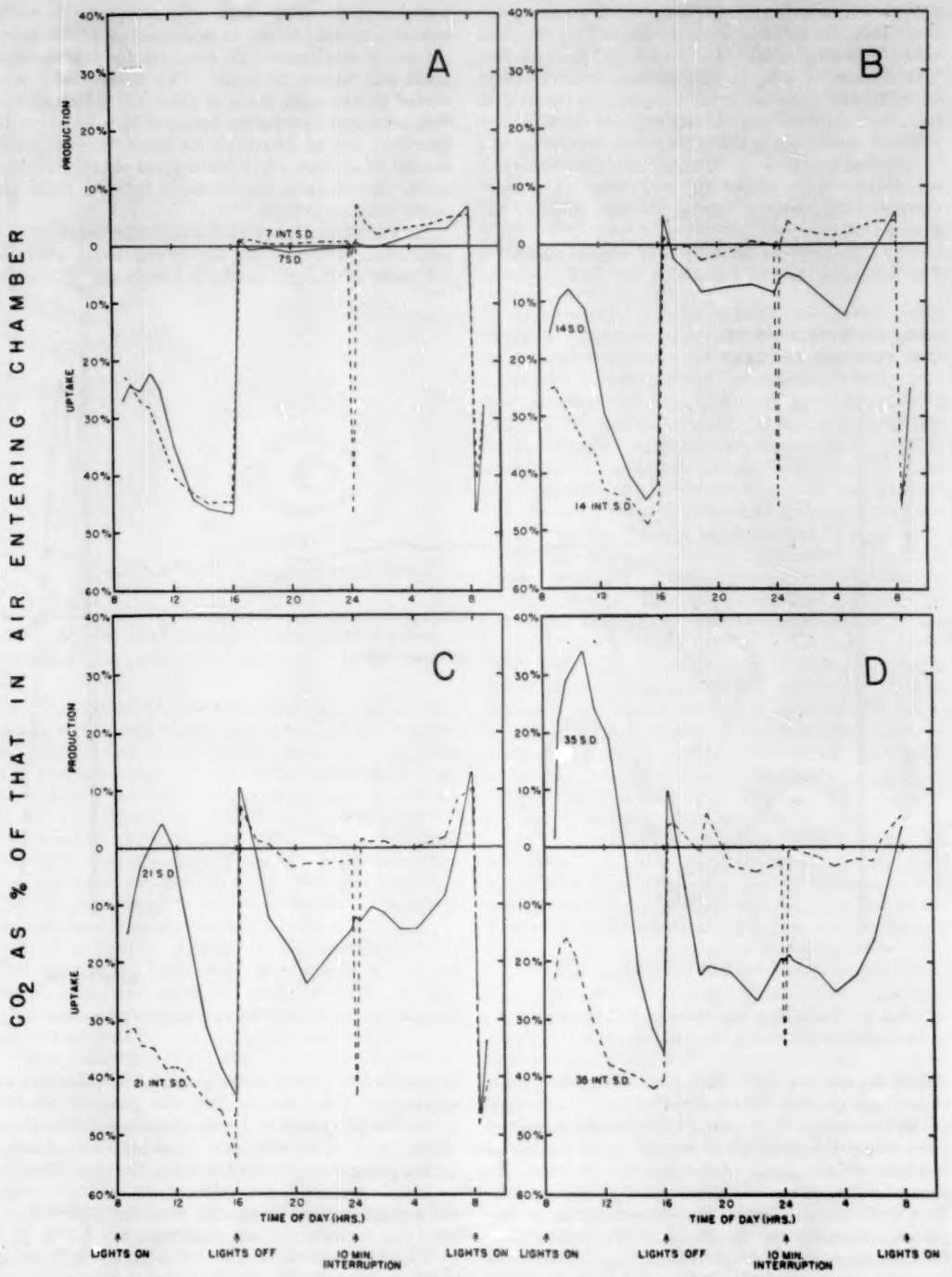

FIG. 6. The diurnal variation in CO_2 metabolism of Kalanchoë after 7 (A), 14 (B), 21 (C), and 35 (D) long nights with (broken lines) and without (solid lines) 10-minute interruptions of the long dark period by 1500 fc. The interrupting light was at midnight (24 hrs.).

absence of dark fixation. However, in plants given short days, the significant production of CO_2 observed at the beginning of the dark period, and the fact that dark fixation of CO_2 rapidly declines after reaching its maximum (just when the stomata are opening in Schwabe's experiments), do not support the idea that stomatal movement is the main factor controlling the CO_2 exchange. It is to be noted also that Schmitz's experiments with plants on very short days show vigorous CO_2 evolution during the first hours of the dark period.

(b) The acceptor for CO_2 is at present unknown. The increasing rate of fixation in the dark might be due to the necessity of a dark reaction for the formation of an acceptor. Since dark fixation is dependent on the preceding light period (fig 6) the precursor from which the acceptor is formed might be carbohydrate or a related photosynthetic product. In plants on long days, the concentration of this material may well become higher, but these plants do not possess, according to the above theory, sufficient of the enzyme system for appreciable CO_2 fixation. In Schmitz's experiments the plants given higher light intensity gave the greater CO_2 fixation in the dark. In the absence of any evidence this explanation seems perfectly plausible.

The cessation of dark fixation could then have two possible explanations. First, it could be due to exhaustion of the acumulated CO_2 acceptor. Secondly, however, it could be due to translocation of the acceptor out of the leaves. To complete the translocation could well require 12 hours. This alternative is supported by the experiment of Bode (2) which showed that increased metabolism occurred in a leaf kept on long day, but so situated that products were translocated to it from a leaf below given short-day treatment. No decision can be made between these two possibilities at present.

2. The CO_2 Burst in Light: The burst of CO_2 production, which occurs for several hours after the beginning of the light period, is presumably due to the destruction of a thermo- or photo-labile substance or substances. Since carbon dioxide is given off, the reaction would appear to be the decarboxylation of an organic acid. This substance is not metabolized away during a long dark period of 48 hours (fig 7). Whether its decarboxylation is due primarily to light or to high temperature cannot be decided from the present data, but must await additional experiments.

There appears to be a direct relationship between the fixation of CO_2 in the dark and its production in the light. In plants kept on long days, neither process occurs appreciably. As one of the processes increases, following successive exposures to short days, so does the other. Taking 43 % uptake as the average level of the CO_2 metabolism during photosynthe-

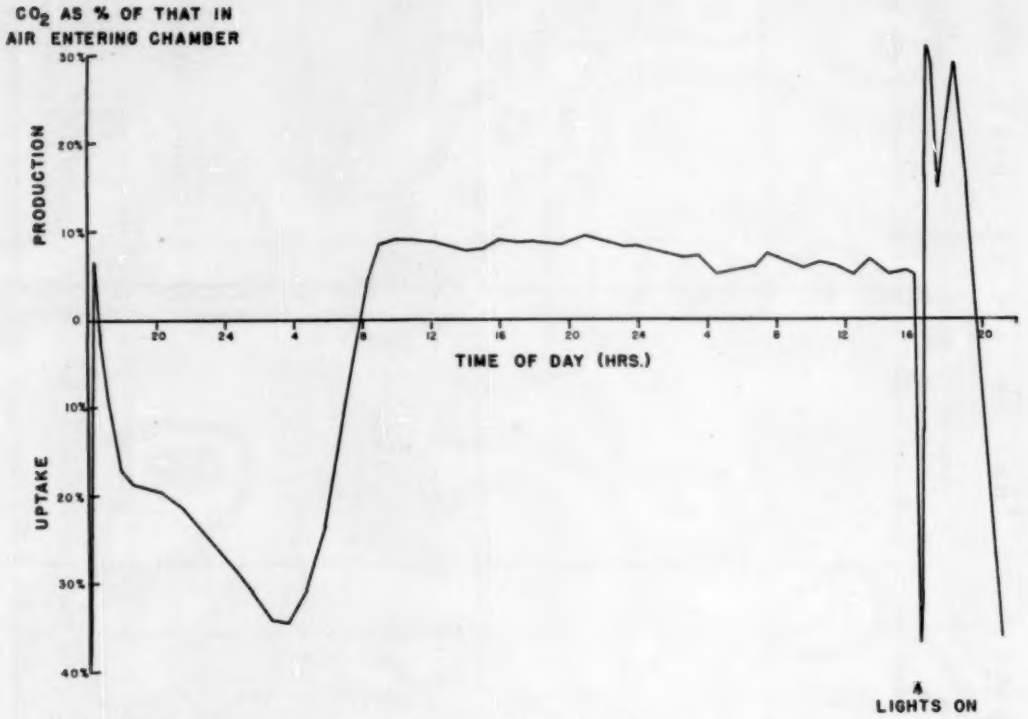

Fig. 7. The CO_2 metabolism of Kalanchoë during a prolonged dark period of 48 hours and 4 additional hours in the light.

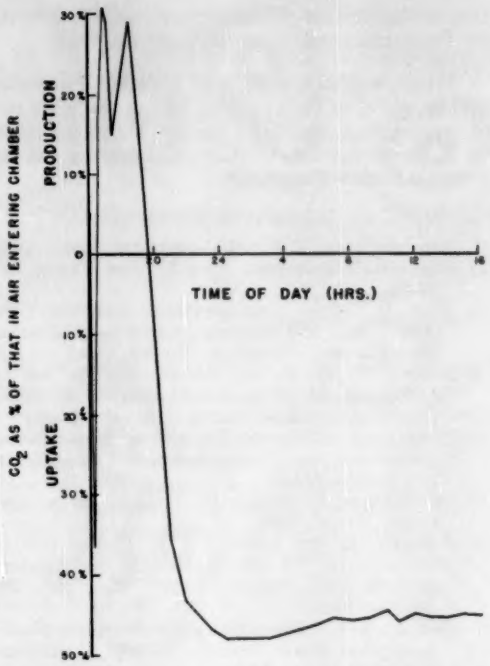

FIG. 8. The CO_2 metabolism of Kalanchoë during a prolonged light period of 24 hours.

sis, the extent of CO_2 production in light can be estimated as the area under the curve (see for instance the 32-short-day curve (fig 3). Similarly if we take 0 % as the average CO_2 production in dark of long-day controls, the dark uptake can be similarly assessed as the area above the curve (33-day curve of fig 2). These areas are almost identical. It is probable, therefore, that the dark fixation directly forms the material decarboxylated in light. It is suggested that this CO_2 burst may be similar in nature to the much smaller burst encountered in Chlorella (6).

3. THE EFFECT OF REPEATED SHORT-DAY CYCLES: The outstanding fact about the fixation of CO_2 in darkness and the associated outburst of CO_2 in light is that the processes are intensified as the number of short days given as pretreatment is increased.

The summation of the effects of repeated cycles of short days could be accounted for by the following hypotheses:

(a) The necessary enzyme system, or coenzyme, for the dark fixation is gradually developed after repeated short day cycles; that is, it requires a long dark period for its formation.

(b) The substrate for the dark fixation, i.e., the CO_2 acceptor, is formed by rapid dark reactions from a precursor which is formed in the light. Hence plants on long-day cycles would contain the precursor but not the enzyme.

The experiments of Bode (2) and of Schmitz (15) discussed above are of further interest in this connection. Bode found that detached leaves from Kalanchoë plants kept in short days had a photosynthetic rate 50 % higher (on basis of leaf area) than those from plants in long days. He also found that the respiration rate was higher, but since these measurements relate to oxygen uptake in the absence of CO_2 their bearing on the present experiment is doubtful. The increased photosynthetic rate, however, should be applicable to our experiments and would mean that the enzyme postulated in (a) above could be concerned also in light fixation of CO_2. The true photosynthetic rate cannot be deduced from the present CO_2 measurements, since it is masked by the liberation of CO_2 in the light.

An important criticism of Bode's experiments must be made. The metabolism of the detached leaves was not studied throughout the cycle but apparently only for relatively brief periods. In view of the large fluctuations in metabolism which we have noted, even within a single light or dark phase, the assumptions of this simplified technique do not seem justified. The same criticism, of course, applies to any method based on integration of the metabolism over long periods. It is for this reason that the data of table I have not been given any detailed interpretation.

Schmitz carried out some experiments with whole plants on 48-hour cycles. Among other things he found that if the light period exceeded 19 hours the plants did not flower. In the interpretation of our results presented above there is no reason why excessively long light periods should in themselves cause loss of the characteristic short-day metabolism. Hence, if indeed the CO_2 metabolism is related to flowering, the CO_2 fixation enzyme, or the precursor, must be gradually destroyed in light. Only if the light phase is below a critical maximum length can some CO_2 acceptor survive, and only thus can the effects of repeated long nights show summation.

4. THE INTERRUPTION OF THE DARK PERIOD: The fact that the interruption of the dark period by brief exposure to light leads to failure of flower induction was shown first by Hamner and Bonner (8) and for Kalanchoë by Harder and Bode (10); it is evidently a general phenomenon in short day plants.

The data in figures 5 and 6 show that, with a brief light exposure of 10 minutes in the middle of the dark phase, plants pretreated with short days fail to show CO_2 fixation in the dark and correspondingly fail to evolve CO_2 in the light. They thus behave, with minor differences, like plants kept continually in long days. The 10 minutes of light interruption is insufficient to release more than a very small fraction of the CO_2 normally appearing in the light phase, and hence this action of light must be exerted, not on the CO_2-containing compound itself, but on the enzyme or substance responsible for producing it. This material, whose concentration builds up in long nights, is destroyed by brief exposure to light during the dark period, but reaches a stable, light-resistant state before the end of the 16 hours of darkness. Its behavior is thus identical with that of the postulated flower-inducing substance (for instance, Gregory, 7).

5. The Relation of the CO_2 Metabolism to Flower Induction: The metabolism of CO_2 investigated in these experiments so closely resembles the effects on flowering induced by similar treatments that some causal relationship seems probable. This is particularly striking in regard to interruption midway in the dark period, which completely prevents flower induction. This was ascribed by Gregory (7) to the formation during the dark period of the precursor of a "flowering hormone." This precursor reached a maximum concentration by the end of the critical dark period, but, if it remained in the leaf, it was destroyed by exposure to light. Thus the interruptions by light had progressively greater effect as the precursor accumulated. However, towards the midpoint of the dark period the precursor was supposed to be translocated from the leaf, so that later interruptions had less and less effect. Before this suggestion can be accepted as holding for the CO_2 acceptor it will be necessary to investigate the effects of interruptions at various times during the dark period. There are many other problems, including the nature of the labile substance and its destruction by light or by heat or both. These are being actively studied.

Summary

The diurnal carbon dioxide metabolism of *Kalanchoë Blossfeldiana* was studied under long days, short days, and short days with interrupted nights. Sodium hydroxide absorption towers and an infra red gas analyzer were used for determining carbon dioxide. A gas-tight chamber is described which encloses only the shoot portion of rooted plants, and enables their study in complete gaseous isolation from the roots. With this apparatus the dark uptake of CO_2 by succulent plants reported by other workers is confirmed for Kalanchoë. It is shown, however, that exposure to repeated short-day cycles increases both the amount and the rate of the net dark fixation of carbon dioxide. The fixation passes through a maximum and may be replaced by a brief period of CO_2 production before the end of the dark period. If the plants are kept in darkness after 16 hours, a steady loss of CO_2 by respiration takes place and continues at a nearly constant level.

Exposure to repeated short days also causes a burst of CO_2 on illumination; the amount of CO_2 evolved parallels the amount of CO_2 absorbed during the preceding dark period. This CO_2 burst also occurs, though only to a very small extent, in plants kept in long days. The two phenomena, i.e., the dark fixation and the CO_2 production in light, are ascribed to the destruction by light or high temperature of a substance, probably an organic acid, produced by CO_2 fixation in the previous dark period. This photo- or thermo-labile substance is not metabolized away in 48 hours of darkness, but appears to require illumination, or high temperature, or both, for its destruction.

Interrupting a long dark period at its mid-point with 10 minutes of light completely inhibits the dark fixation of CO_2 and also the associated CO_2 production on illumination. Various mechanisms to account for the results obtained are critically discussed.

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THE ABSORPTION OF INORGANIC IONS BY CHLORELLA PYRENOIDOSA [1,2]

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In the course of experiments on the effect of radioactive elements on the growth of *Chlorella pyrenoidosa*, Porter and Knauss (9), determinations were made of the content of a radioactive element in algal cells when the ratio of that element to its stable isotope was kept constant, but the total concentration of the two isotopes in the solution was varied. Calculations were then made, assuming no isotope effect, of the total contents of the two isotopes in the algal cells. These determinations were made for the elements P, S, Ca, Fe, Mn, Zn, Cu, and Sr.

A limited amount of information is available on the effect of the nutrient concentration of an element on the uptake of that element by algae. Scott (11) has reported that the ratio of uptake of calcium and magnesium by Chlorella depends upon the ratio of these two elements in the nutrient solution. Scott (12) has also studied the behavior of phosphate-deficient cells during a restoration period when adequate phosphate was supplied to the nutrient medium. He found the phosphate uptake to be proportional to the nutrient concentration only at levels that were limiting for growth.

More information on the effect of nutrient concentration of an element on its uptake has been obtained through studies with higher plants and diatoms. Beckenbach et al (1), in making a statistical analysis of the relationship between the ionic content of the culture solution and the element content in corn plant tissues, found the most important single factor affecting the absorption and accumulation of an element in plant tissues to be its absolute concentration in the nutrient solution. Similar results were reported by Tidmore (14) on corn, sorghum, and tomatoes, and by Beeson et al (2) on tomato plants. Recently, Rediske and Selders (10) reported the uptake of strontium by plants to be proportional to the concentration of this element in the nutrient solution. Goldberg et al (5), working with diatoms, found the phosphate uptake to be proportional to the concentration in the medium over the range 0.5 to 3.5 μg/l. Similar findings were reported by Ketchum (7).

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[2] This paper is based on work performed under Contract No. W-31-109-Eng-52 for the Atomic Energy Commission.

EXPERIMENTAL METHODS

The strain of Chlorella used was American Type Culture No. 7516. Inocula to use in experiments were obtained by the same procedure reported previously (9).

Seven and one half ml of nutrient solution (KNO_3, 3.0 gm/l; $MgSO_4 \cdot 7\,H_2O$, 4.8 gm/l; $Ca(NO_3)_2$, 1.0 gm/l; KH_2PO_4, 3.0 gm/l; $MnSO_4 \cdot H_2O$, 2.0 mg/l; H_3BO_3, 2.0 mg/l; $CuSO_4 \cdot 5\,H_2O$, 0.2 mg/l, and $ZnSO_4 \cdot 7\,H_2O$, 0.2 mg/l), less the element being investigated, were added to 20×150 mm culture tubes. Varying amounts of the element being studied and the appropriate amounts of water were added to each tube. After sterilization each tube received 0.2 ml of a sterile iron solution (1 gm $FeSO_4 \cdot 7\,H_2O$/500 ml), 1.0 ml of inoculum (an optical density of 0.60 at 660 mμ/18 mm tube, approximately 17×10^6 cells/ml) and amounts of a solution containing a radioactive isotope of the element under study to yield a final specific activity of one mc/gm of that element. In experiments on the absorption of copper the specific activity was 3 to 8 mc/gm of element. Radioisotopes used were P^{32}, S^{35}, Ca^{45}, Fe^{59}, Mn^{54}, Cu^{64}, Zn^{65}, and Sr^{90}. The final volume of solution was 18.2 ml, and the final pH was 4.2 to 4.6. Duplicate tubes were set up for each concentration of the element under study. Control tubes, lacking in radioactivity, were also inoculated in each experiment.

The cotton plugs of the tubes were replaced with sterile 0.5 mm capillary tubing wrapped in cotton. The cells were then aerated through these tubes at a rate of approximately 100 ml per minute with a mixture of 8 to 9 % CO_2 in air. The gas mixture was saturated with moisture by passing through water. Light intensity (white fluorescent) incident to the tubes during the growth period was 300 to 500 fc.

After the growth period, usually 72 hours, the suspensions of cells were made to a common volume such that light absorption readings could be made. These were then made on the cell suspensions in 18 mm tubes at 660 mμ with a Beckman Model DU Spectrophotometer. From these readings the dry weight for each sample was determined by reference to a previously determined relationship between dry weight and optical density (fig 1).

The procedure of a typical experiment in which

the relationship between dry weight and optical density was determined was as follows. A liter Erlenmeyer flask containing 500 ml of nutrient medium was inoculated with 32 ml of a washed suspension of Chlorella that had an optical density of 0.61 in an 18 mm tube at 660 mμ, a cell number of 16.6×10^6/ml and a dry weight of 0.15 mg/ml. The solution was aerated with a mixture of 8 to 9 % CO_2 in air at such a rate as to prevent sedimentation of the cells.

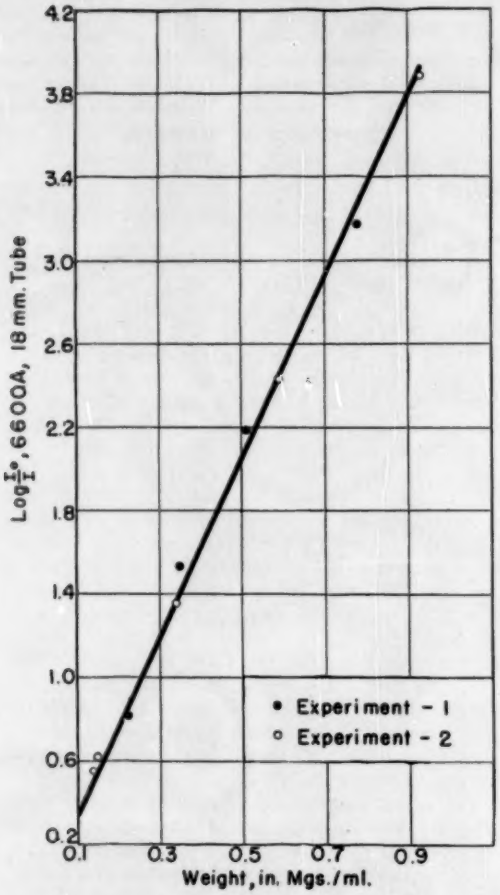

FIG. 1. The relationship between optical density and dry weight of Chlorella.

Light intensity at the surface of the solution was approximately 200 fc. Temperature was approximately 25°C. Daily, after inoculation, aliquots were taken for dry weight and light absorption determinations. When the optical density of the solution exceeded 1.00, the suspension was diluted to an optical density of approximately 0.60. Weight of the samples was determined as follows. Twenty-five ml of the original suspension was centrifuged at 10,000 × g, then 3 times resuspended with distilled water and centrifuged. The residue was transferred with a small amount of water to a weighed boat. The sample was dried on a microheater with a stream of nitrogen, then in a vacuum oven at 62°C for 2.5 hours. Weighings were made on an analytical balance. The data of figure 1 are those obtained in 2 experiments.

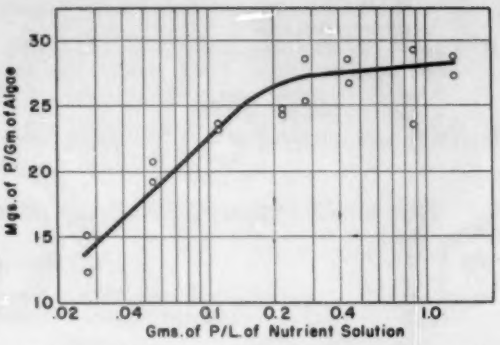

FIG. 2. The phosphorus content of Chlorella grown at different nutrient concentrations of phosphorus.

Radioactive algal cells and nutrient solution were transferred (1-ml aliquots) from each tube on which light absorption readings were made to 1-inch stainless steel plates for counting. Orthodox beta counters employing a Geiger-Müller tube were used for counting radioactive P, S, Ca, Fe, Cu, and Sr. A scintillation counter was used to count radioactive Zn and Mn. Calculations were then made, using the appropriate factors for geometry, self-absorption, etc, of the radioactivity and the specific activity of the solution.

The remaining cells in each tube were centrifuged at aproximately 10,000 × g for 2 minutes, and the supernatant solution was decanted. The cells were resuspended in 10 ml of nutrient solution that contained the element in question at a concentration of 0.1 % (except iron which was held at a concentration of 0.04 %). The cells were again centrifuged, the supernatant solution decanted, and the cells suspended in a volume of 10 ml. A 2.0 ml aliquot from each suspension was transferred to a 1-inch plate for counting. Calculations were then made of the

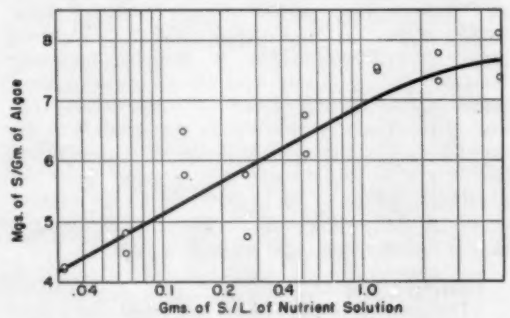

FIG. 3. The sulfur content of Chlorella grown at different nutrient concentrations of sulfur.

TABLE I

A SUMMARY OF THE GROWTH AND ELEMENT ABSORPTION BY CHLORELLA

ELEMENT	MASS OF CELLS AFTER 72 HOURS	k VALUES $(y = kx + C)$	RANGE IN NUTRIENT CONCENTRATION	RANGE IN CONCENTRATION IN CHLORELLA	NUTRIENT ELEMENT REMOVED BY CELLS
	gm/l		*mg/l*	*mg/gm*	*%*
Fe	0.389 ± 0.016[a]	2.19 ± 0.30	0.104–13.31	0.237 –34.53	85.04 ± 10.82
Mn	0.285 ± 0.021	2.49 ± 0.12	0.165–10.56	0.241 –25.95	70.45 ± 6.82
Ca	0.325 ± 0.025	0.0085 ± 0.0016	4.28 –547.8	0.059 –4.00	0.28 ± 0.06
Sr	0.942[b] ± 0.037	0.0075 ± 0.0010	0.550–70.4	0.0044–0.507	0.70 ± 0.09
Cu	0.434 ± 0.024	0.65 ± 0.17	0.017–0.281	0.0082–0.337	27.65 ± 12.02
Zn	0.702[c] ± 0.099	0.14 ± 0.04	0.021–0.856	0.0038–0.093	9.27 ± 3.44
P	0.576 ± 0.041	16.7[d]	27.5 –200	13.5 –23.4	24.4 — 11.9[e]
P	0.576 ± 0.041	.....	200 –1375	23.4 –27.6	.....[f]
S	0.969[b] ± 0.104	1.77[d]	31.9 –1020	4.23 –7.45	14.8 — 0.75[e]
S	0.969[b] ± 0.104	.....	1020 –4078	7.45 –7.67	.....[g]

[a] All ± values are standard errors at 95 % confidence level.
[b] 120 hour growth period.
[c] 96 hour growth period.
[d] $y = k \log x + C$; for (P), $y = 16.7 \log x - 10.53$; for (S), $y = 1.77 \log x + 1.57$.
[e] Range of percent of element removed.
[f] Concentration in cells remained constant at approximately 27.5 mg/gm above a nutrient concentration of 200 mg/l.
[g] Concentration in cells remained constant at approximately 7.5 mg/gm above a nutrient concentration of 1020 mg/l.

radioactivity and the total content of the stable and radioactive isotopes in the cells. Corrections were made for the decay of the radioisotopes within the algal cells during the time elapsing between the determination of specific activity of the nutrient solution and the radioactivity of the cells.

In experiments with Sr^{90}, which forms a radioactive decay equilibrium mixture with Y^{90}, decay curves were obtained for the radioactivity in cells and in cells plus nutrient solution. Counts obtained at equilibrium, 30 days after harvest of the cells, were used in calculating the radioactivity and the amount of Sr present in the cells.

In experiments with Fe^{59}, carrier iron was supplied as either Fe^{++} or Fe^{+++}. Absorption of this element by the algae was the same in either case.

RESULTS AND DISCUSSION

The results of the various experiments are presented in figures 2 to 9 and in table I. These figures, and the table, show that the amount of an element absorbed by the algae is a function of the element and its concentration in the nutrient solution. The

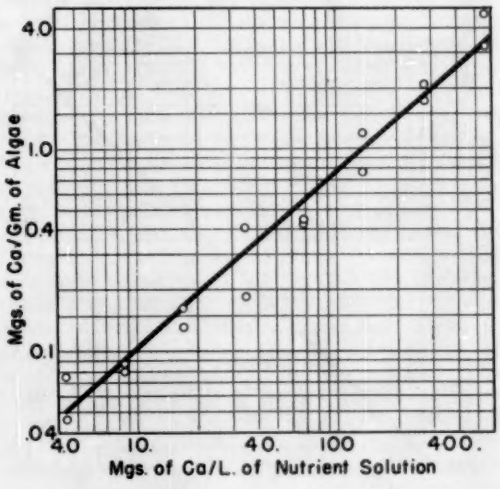

FIG. 4. The calcium content of Chlorella grown at different nutrient concentrations of calcium.

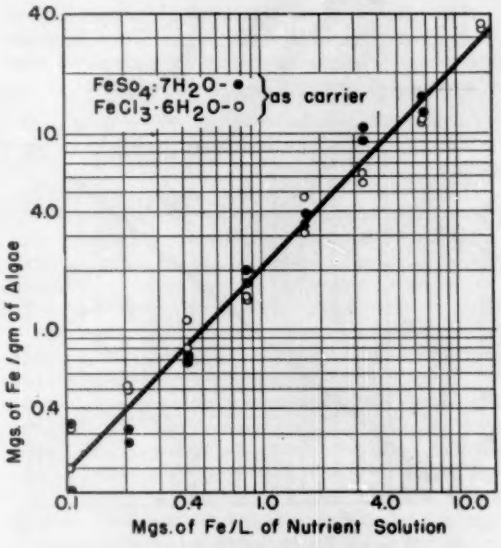

FIG. 5. The iron content of Chlorella grown at different nutrient concentrations of iron.

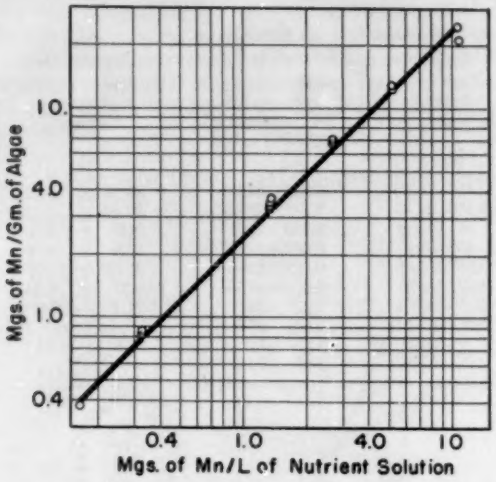

FIG. 6. The manganese content of Chlorella grown at different nutrient concentrations of manganese.

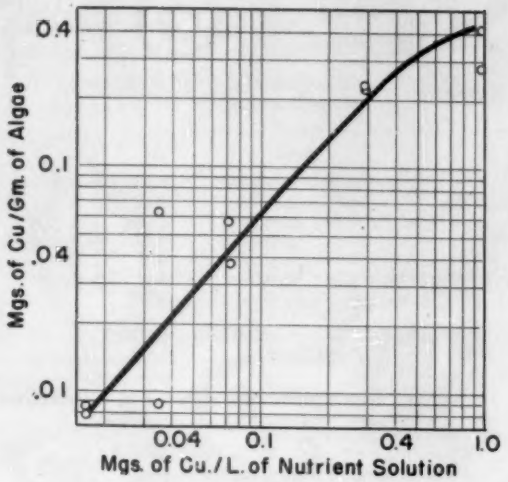

FIG. 8. The copper content of Chlorella grown at different nutrient concentrations of copper.

absorption of iron and manganese from the nutrient solution by Chlorella was much greater per equal quantity of element than it was for the other elements used. In fact, since the absorption of these elements was so great, we are inclined to believe that most of the iron and manganese was adsorbed on the cell surfaces, in complexes which did not exchange with nutrient element during the washing procedure, rather than absorbed into the cell. Also noteworthy are the large quantities of phosphorus that were absorbed into algal cells, and the small proportions of nutrient sulfur, calcium and strontium that were absorbed by the cells.

The effect of concentration of a nutrient element on the absorption of that element by Chlorella is also interesting. Phosphorus and sulfur contents of algal cells remained constant at the higher levels of nutrient concentration of these elements. At lower levels of nutrient concentration, absorption into the cells was equal to a factor (k) times the log of the nutrient concentration plus a constant, (C). Absorption of other elements into the algal cells was equal to a constant (k) times the nutrient concentration plus a constant, (C).

Growth of the cells (table I) in experiments with different elements varied, but the growth within an experiment did not indicate limiting or toxic element concentrations. Whatever caused variation in growth between experiments was common to each tube in an experiment. Concentrations of elements within cells at growth-limiting levels of nutrient concentration were not determined.

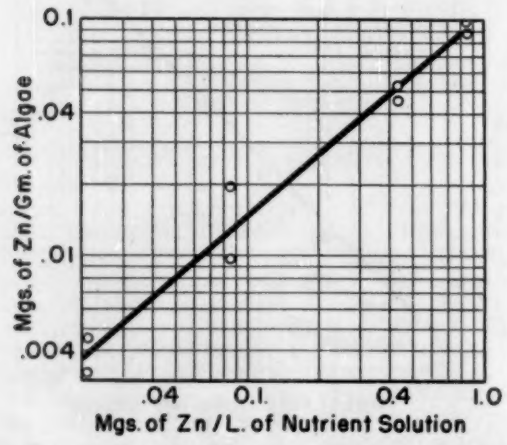

FIG. 7. The zinc content of Chlorella grown at different nutrient concentrations of zinc.

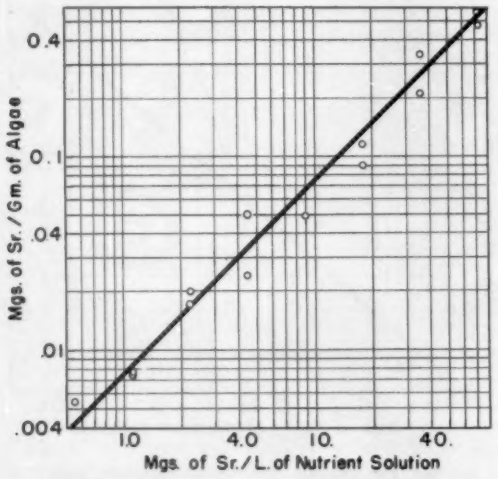

FIG. 9. The strontium content of Chlorella grown at different nutrient concentrations of strontium.

The tremendous differences in absorption of elements by Chlorella suggests that absorption (and adsorption) pathways of elements entering the cell may be quite different. If so, then a considerable number of enzymes would be involved in elemental absorption, and a considerable variation in elemental absorption could be effected by varying factors which affect the rates of reaction of these enzymes. A factor of paramount importance in determining the amount of an element absorbed by cells is the concentration of this element in the nutrient solution; and more important is the fact that the uptake of elements by Chlorella has been related mathematically to nutrient element concentration, other factors kept constant. An example of the remarkable precision with which the forces within a cell work, and the effect of changing nutrient concentration on this system, is seen in the uptake of calcium and strontium by Chlorella cells. The k values for these elements, which are quite similar chemically, are the same even though nutrient concentrations of these elements were quite different.

Several sources of error exist in tracer experiments which, like those reported in this paper, are designed to determine the actual content of an element in cells. These include the possibility of (1) differences in valence state or compound between the tracer and the carrier, (2) radiochemical impurities, (3) an isotope effect, (4) selective adsorption of radioisotope on the walls of the container, (5) exchange between the radioisotope of the cells and the carrier of the solution during the washing process, and (6) adsorption of the radioisotope on the surface of the cells. By far the most difficult sources of error to cope with are (4), (5), and (6). In our experiments (4) was found to be unimportant. Activity counts made on the culture solution, when corrected for decay, checked quite closely with the activity added. Considerable disagreement exists in the literature as to the importance of (5) and (6). This difference seems to be attributable to (1) the method (tracer or non-tracer) used and (2) the quantity of the element in the nutrient solution. Scott (13), using non-tracer methods, found that mineral constituents were not removed by suspending Chlorella cells in distilled water. He also found that potassium is not removed by centrifuging and resuspending the Chlorella 6 times in potassium-free Detmer's medium. Goldberg (5), using tracer methods, found that at a minimal concentration in the nutrient solution (approximately 0.5 μg P/l), the phosphorus present in diatom cells is strongly bound and is not removed by permitting the cells to stand three weeks in suspension. He also found, however, that when the cells were grown at higher concentrations of phosphorus, washing removed a considerable portion of the element. The latter results are generally in agreement with those of Gest and Kamen (4) who found, by radiochemical methods, that Chlorella cells grown in a solution containing the amount of phosphate recommended for optimum growth, contain excess inorganic or highly labile phosphate. This phosphate can be washed from the cells, but the phosphate present in cells grown in a medium of low phosphate content cannot be so removed.

As a check on the effect of washing cells with a stable isotope on the removal of the radioisotope, algae were grown in the presence of several levels of phosphorus and then washed 0, 1, 2, and 3 times with a solution containing 0.1 % of stable phosphorus. The results presented in table II indicate that 1 wash did not remove appreciable quantities of P^{32} from cells grown in the presence of low and medium concentrations of phosphorus. Some P^{32} was removed from those cells grown at the higher levels of nutrient phosphorus. Additional washes did not materially change the phosphorus content of the cells, except at 3 lower levels of nutrient phosphorus content. The procedure of a single wash of the cells with a solution of the nutrient under study was then arbitrarily adopted as one which would give most accurate values for elemental content within the cell structure.

TABLE II

THE PHOSPHORUS CONTENT OF ALGAL CELLS WASHED 0 TO 3 TIMES WITH A SOLUTION CONTAINING 0.1 % OF PHOSPHORUS

NUTRIENT CONC. OF P	P CONTENT, MG/GM ALGAE			
	NUMBER OF WASHES			
MG/L	0	1	2	3
2.75	7.5	7.0	5.4	5.0
5.50	16.6	15.8	14.5	10.6
11.00	38.1	38.0	34.0	25.7
22.00	46.5	44.4	42.8	28.8
44.00	36.3	33.5	34.0	32.8
88.00	39.1	37.8	33.0	33.2
176.0	39.9	34.5	35.4	33.5
352.0	35.2	21.7	29.2	29.4
704.0	43.6	36.4	37.5	33.4
1408.0	43.4	27.1	26.0	27.8

In comparing the values reported in this paper with those of others it was noted that our values for calcium and iron agree with those found in the literature (8, 11, 13). Our values for phosphorus are, however, 10 to 100 times those reported by Scott (11, 12, 13), but they are within the range of 1 to 4 times those reported for bean plant leaves (3). No explanation, other than a difference in method, is immediately apparent for the difference between our results and those of Scott.

The fact that the absorption of an element by algae is a function of the concentration of that element in the nutrient solution has importance in research designed to improve the control of hazards from radiations and radioactive elements. By controlling the quantity of element in the nutrient solution and the ratio of radioisotope to stable isotope, it should be possible to obtain algal cells which will have the same quantity of radioactivity per mass of algal cells, but a difference in the number of disintegrations in vitally important compounds (particu-

larly enzymes and genes) of the cells. This is essentially the same technique as employed by Hungate and Mannell (6) in studies with Neurospora. It should then be possible through subsequent studies of growth rate, numbers of killed cells, and numbers of mutations, to determine the relative hazard of transmutation of radioelements in algae.

Summary

The isotope method was used to determine the quantities of an element present in Chlorella when the nutrient concentration of that element was varied. Elements used in these studies were P, Ca, S, Fe, Mn, Zn, Cu, and Sr.

The absorption, by the algae, of all elements except P and S was directly proportional to the concentration of that element in the nutrient solution. The quantities of P and S in the algal cells were constant when the cells were grown in the higher nutrient concentrations of these elements.

A brief discussion is presented of: 1) the sources of error existent in the isotope method of determining the elemental content of algae; 2) the effect of the nutrient concentration of an element on its absorption by algae.

The authors wish to acknowledge the technical assistance of Mrs. D. G. Watson. The assistance of members of the Biological Services Unit of this Section in preparing and calibrating solutions of the isotopes and in counting samples is also gratefully acknowledged.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON CATION ABSORPTION BY BARLEY ROOTS [1,2]

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Hoagland and Broyer (2) working with excised barley roots and Arnon, Fratzke, and Johnson (1) working with intact plants demonstrated a pronounced effect of certain concentrations of hydrogen ion on mineral absorption.

In a former research of this laboratory dealing with the absorption of potassium by barley roots (3), it was postulated that the absorption process involves the following reversible reaction,

$$HR + K^+ \rightleftarrows KR + H^+$$

where HR represents a metabolically produced cationic binding substance and KR, a labile organic complex of potassium. To a large extent, the experiments were designed to study the reverse of this reaction; that is, the effect of hydrogen ion on the absorption of potassium by roots.

In a number of respects the results were con-

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[2] This paper is based on work performed under contract No. AT-(11-1)-34, project 5, with the Atomic Energy Commission.

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sistent with the postulated absorption reaction. As the H^+ concentration of the culture medium was increased, increasing amounts of potassium were lost by the roots to the medium. Also, a pronounced competition between K^+ and H^+ in absorption was established.

Under conditions favoring the absorption of hydrogen ion and presumably following the absorption of hydrogen ion, the roots underwent a series of irreversible changes. For example, when barley roots were immersed for two hours in 0.001 *N* HCl solution at room temperature, the major fraction of their K, Ca, P, and soluble N contents was lost. Moreover, the ability of the tissues to reabsorb potassium was virtually eliminated.

These drastic effects of H^+ on the tissues could be offset in large measure by rather low concentrations of calcium ion, and also, to a small extent, by Na^+.

It seemed plausible that the effect of sodium might be the result of a competition between H^+ and Na^+ in absorption. However, a similar explanation for the effect of calcium did not appear reasonable since the tissues under study absorb Ca^{++} very slowly.

The present paper reports further study of the effect of hydrogen ion on potassium absorption in root tissues and of modifications of the effect by Ca^{++} and other polyvalent cations.

Experiments

The experimental material used was excised barley roots (Sacramento variety, 1951 crop) cultured and prepared as described previously (3). The proportion of one gram of root tissue per liter of solution was maintained throughout in order to minimize concentration changes. The experiments were conducted at 26°C, and the periods of pretreatment and absorption were three hours each.

The first experiment tested the effects of various monovalent and polyvalent ions at different values of pH on the subsequent ability of the tissues for potassium absorption. Duplicate, 15-gram batches of roots were each placed in 15 liters of an 0.001 *N* solution of the chloride salt of the element at the desired pH and the suspension was aerated for a period of three hours. Following the pretreatment, the duplicates were washed and one was set aside for potassium analysis. The other was placed immediately into 15 liters of 0.001 *N* KCl (pH 5) and allowed to absorb for three hours with aeration. The roots were then washed and analyzed for potassium. The losses of potassium as a result of the pretreatments are presented in table I. The absorption of potassium from the 0.001 *N* KCl solution as a function of the pH of the pretreatment solution is given in figure 1 for each of the elements investigated. The potassium absorption is presented as milliequivalents of K^+ absorbed per 1000 gm of fresh roots. It will be noted that these experiments include a pretreatment with distilled water adjusted to the different pH values with HCl.

The second set of experiments was designed to study the effects of various monovalent and polyvalent cations on potassium absorption at different values of pH. Weighed batches of roots were placed in 0.001 *N* KCl solutions which were also 0.001 *N* in the chloride salts of the desired elements and adjusted to the desired values of pH. For comparison, a series of batches were placed in pure 0.001 *N* KCl solutions adjusted to the required pH with HCl. The roots were allowed to absorb for three hours with aeration. The tissues were then washed and analyzed for potassium. In these experiments the potassium content of the original root material was 16.5 mEq per 1000 gm fresh roots. The potassium absorbed as a function of the pH of the culture solution is presented in figures 2 and 3. In figure 2 the effects of monovalent cations are presented and the effects of polyvalent cations are shown in figure 3. In both figures the absorption from the pure KCl solutions is presented for comparison. A positive value for the absorption corresponds to a gain of potassium and a negative value corresponds to a loss.

Discussion

Table I reveals that drastic changes in the potassium content of the roots may occur as a result of pretreatment with solutions at different values of pH. Also, these effects may be greatly influenced by the species of cations present in the solution. At pH 1 and 2, essentially all of the potassium is lost from

Table I

Percentage Change in Potassium Content* during Pretreatment** with 0.001 N Chloride Solutions at Various pH Values

Pretreatment	pH					
	1	2	3	4	5	6
H_2O	−98.6 %	−98.6 %	−90.6 %	−34.3 %	− 2.36 %	− 2.05 %
Li^+	−98.9	−97.0	−95.2	−69.4	−24.35	−14.15
Na^+	−99.3	−99.0	−92.3	−29.8	−14.93	−11.10
Al^{+++}	−99.3	−99.0	−24.85	−12.10	....	....
Ca^{++}	−99.3	−99.0	−52.5	+ 1.47	− 7.88	− 2.05
La^{+++}	−98.4	−97.3	−22.6	−14.34	−13.00	−11.55
Ce^{+++}	−98.3	−97.0	−25.9	−11.1	−17.03	− 6.69

* Potassium content of original roots = 16.95 mEq per 1000 gm fresh weight.
** Period of pretreatment: 3 hours.

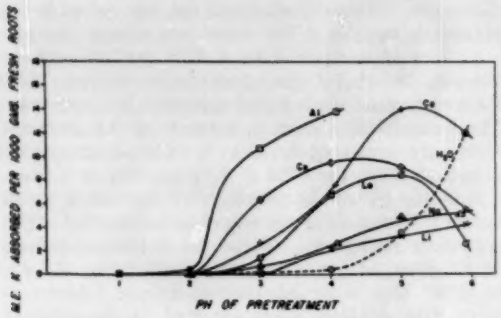

FIG. 1. The absorption of potassium from 0.001 *N* KCl solution (pH 5) as a function of the pH of the pretreatment solution. Each curve gives the K absorption following pretreatment with solutions of the chloride salt of the indicated cation. These solutions were adjusted to the different values of pH with HCl. The period of pretreatment and the subsequent potassium absorption period were of three hours duration. The dotted curve shows the effects of pretreatments with distilled water adjusted to the desired pH values with HCl.

the tissues, regardless of the nature of the salts present. At pH 3, major differences related to the kind of salt present begin to appear. At this hydrogen ion concentration, the losses in the presence of the chloride salts of Al, La, and trivalent Ce are markedly less than those in the presence of the salts of the other cations and in the presence of HCl alone. In the pH range, 4 to 6, the effect of the presence of calcium is notable. Here the losses in the presence of this ion are less than those in the presence of the other cations. At pH 4, the loss in distilled water (i.e., in dilute HCl) is considerable; at pH 5 and 6, the losses are probably insignificant.

It is evident from figure 1 that the various pretreatments had pronounced effects on the capacities of the tissues for subsequent K absorption at pH 5. The effects of the distilled water (dilute HCl) pretreatments are particularly interesting. In view of the fact that unpretreated roots absorbed 13.7 mEq K per 1000 gm under these conditions, it is remarkable that pretreatment in water at pH 5 to 6 should effect so markedly the absorption capacity of the roots. Evidently this is a specific hydrogen ion effect since the reduced K absorption cannot be correlated with the loss of potassium during pretreatment. Although this effect is readily reproducible for a particular batch of seed, the magnitude of the phenomenon may vary widely with the variety of barley used and even with the source of seed of the same variety.

It is apparent from figure 1 that the polyvalent cations have a pronounced protective effect in the lower pH range of pretreatment. The monovalent cations, Li^+ and Na^+, also have a small protective effect in this range although in the pH range 5 to 6 they cause a reduction in the capacity of the roots for K absorption. The protective action of the polyvalent cations rises with pH to a maximum in the neighborhood of pH 5 above which it may fall off abruptly.

Figure 2 shows that the presence of monovalent cations has little effect on the point (about pH 4) where there is no gain or loss of potassium. In the pH range above this critical point, the monovalent cations depress the absorption of K. In the range below the point, they exert a small protective action. Although the reduction in absorption above the critical point may be at least in part due to ion competition, this cannot be the whole explanation since pretreatment with the monovalent cations reduces subsequent potassium absorption.

Figure 3 reveals that polyvalent cations effect a pronounced shifting of the critical point. The critical point, with or without the presence of monovalent cations, is situated at about pH 4.25. When the polyvalent cations are present, however, the point is in the neighborhood of pH 2.5. In the lower pH range the effect of Ce^{+++} is similar to that of the other ions. At higher pH, however, Ce^{+++} depresses the absorption of K. Most likely this is a toxicity phenomenon. It is evident from figure 3 that Al^{+++} exerts the greatest protective action. However, it was not possible to examine the behavior of this ion above pH 4 because of the hydrolysis of $AlCl_3$. It should be noted that even in the pH range 5 to 6 the absorption of potassium in the presence La^{+++} and Ca^{++} is considerably greater than in KCl alone. The data

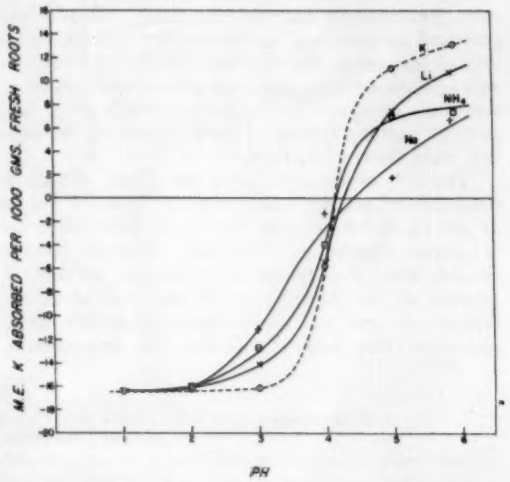

FIG. 2. The absorption of potassium from solutions 0.001 *N* in KCl and 0.001 *N* in the chloride salt of the indicated cation as a function of the pH of the culture solution. The culture solution was adjusted to the desired pH with HCl. The dotted curve indicates the absorption from 0.001 *N* KCl solutions adjusted to different pH values with HCl. A negative value for the absorption represents a loss of potassium. The period of absorption was three hours. The potassium content of the original roots was 16.51 mEq per 1000 gm fresh weight.

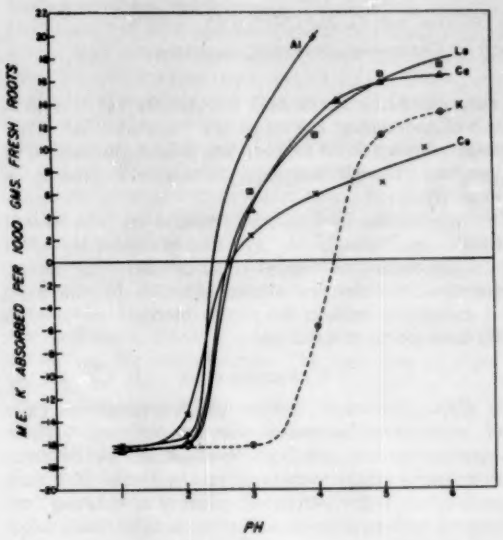

FIG. 3. The absorption of potassium from solutions 0.001 *N* in KCl and 0.001 *N* in the chloride salt of the indicated cation as a function of the pH of the culture solution. The culture solution was adjusted to the desired pH with KCl. The dotted curve indicates the absorption from 0.001 *N* KCl solutions adjusted to different pH values with HCl. A negative value for the absorption represents a loss of potassium. The period of absorption was three hours. The potassium content of the original roots was 16.51 mEq per 1000 gm fresh weight.

suggest that the stimulating effect of polyvalent cations on the absorption of monovalent ions reported by Viets (5) may be due at least in part to the interrelation of the polyvalent cations and hydrogen ion. If, for example, the effect of the presence of Ca^{++} were determined at pH 4.5, it might be interpreted as a large stimulating effect. However, the data of figure 3 indicate that the observed stimulation may be due partly to a shift in the critical point. Whether the stimulating effect of Ca^{++} at pH 6 and above is the result of an interrelation with H^+ ion requires additional investigation.

In a previous publication (4) it was suggested that the effect of Ca^{++} on potassium absorption could be explained in terms of the reaction,

$$HR + K^+ \leftrightarrows KR + H^+$$

if Ca^{++} ion effects a decrease in the concentration of KR, by chemical or physical means, and thus shifts the absorption reaction to the right. The data of this paper are consistent with this postulate since such a shifting of the equilibrium to the right should decrease the pH sensitivity of absorption. This has been observed in the presence of Ca^{++} and other polyvalent cations.

SUMMARY

Potassium loss and gain in excised barley roots has been investigated in the pH range 1 to 6.

Pretreatment of the roots with pure HCl solutions results in a pronounced loss of potassium from the tissues below pH 5. This loss is offset in large measure by the presence of the polyvalent cations, Ca^{++}, Al^{+++}, La^{+++}, and Ce^{+++}.

Pretreatment with pure HCl solutions below pH 6 results in a marked reduction in the capacity of the roots for subsequent potassium absorption. However, small concentrations of Ca^{++}, Al^{+++}, La^{+++} or Ce^{+++} in the pretreatment solution greatly lessens this effect below pH 5. A small protective action of Li^+ and Na^+ was noted in the same pH range.

The rate of K absorption from 0.001 *N* KCl falls off rapidly below pH 6. Below pH 4.25 the rate is negative, that is, potassium is lost from the tissues. Small concentrations of Ca^{++} and La^{+++} in the culture solution showed a pronounced stimulating effect on K absorption for the pH range 2 to 6. Al^{+++} and Ce^{+++} ions also had a large stimulating effect in the pH range 2 to 4.

As a result of this stimulating effect, the rate of K absorption in the presence of the polyvalent cations did not become negative until below pH 2.5.

The stimulating effects of polyvalent cations on the absorption of monovalent ions, reported by Viets (5), may be due at least in part to the interrelation of the polyvalent cations and hydrogen ion.

A possible explanation of the observed effects of polyvalent cations is presented.

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ON THE BIOSYNTHESIS OF CAROTENOIDS[1]

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The carotenoid mixtures in diverse plant tissues and the proportions in which individual components occur show great variation. As yet no general relationship between these compounds and their relative amounts can be clearly demonstrated. Goodwin (1) has recently reviewed the possible pathways of synthesis which at this stage are not mutually exclusive. A "sequential" pathway, postulated by Porter and Lincoln (10), involves a series of step-wise dehydrogenations from tetrahydrophytoene, $C_{40}H_{76}$, through phytofluene to lycopene, $C_{40}H_{56}$. The alternative is a "parallel" pathway in which the synthesis of the majority of the various C_{40} compounds depends upon the availability of a particular fragment which in turn determines the end product. In this case it is generally assumed that after formation of the C_{40} chain, interconvertibility is limited.

As experimental material, tomatoes (4, 7, 10) and the mold *Phycomyces blakesleeanus* (2, 8, 9) have certain obvious advantages and less obvious disadvantages. By hybridization, segregation, and backcrossing, the gross effect of a single gene difference in the tomato may be laboriously ascertained without necessarily explaining the mechanism whereby the effect was induced. Treatment of the mold culture with such substances as diphenylamine (2), β-ionone (9), or methylheptenone (8), markedly influences the carotenoids elaborated. Again, an unambiguous interpretation is not yet possible. Tracer studies with labeled ionone have had the disadvantage that considerable activity is found in petroleum ether extracts of the mold, not associated with the β-carotene *after* crystallization. Phytofluene is an oil and there is some uncertainty as to whether ζ-carotene has been crystallized. In any event, purification at the level of 200 to 500 μg with the aid of carrier has so far only been achieved with β-carotene, and it remains unresolved as yet whether phytofluene is a precursor of ζ-carotene.

While continuing these studies, we routinely examine new experimental material that may be available. Thus, Khan and Mackinney (5) showed recently that in both pink- and white-fleshed grapefruits, the ζ-carotene and phytofluene are at a low level. Lycopene and β-carotene are absent. Sporting of the Marsh white grapefruit yields fruit with high β-carotene and low lycopene; then further sporting yields fruit with unchanged β-carotene content and increased lycopene.

In our view, many such facts must be accumulated before a general pattern of carotenoid synthesis will find acceptance.

In this paper, we report on carotenoid differences in a new source-material, the common yellow pansy, *Viola tricolor*. This contains predominantly a complex mixture of xanthophylls from which violaxanthin was isolated by Kuhn and Winterstein (6). A variant of the yellow, known as the "apricot *Viola*," obtained from a local nursery has a high proportion of lycopene, though xanthophylls are still present in quantity.

In addition, we include reports of two recent studies on *Phycomyces*. The first evaluates the effect of light on ionone-treated cultures (5). The second demonstrates the preferential selection by the mold of non-sugar carbon from the medium we use, in its elaboration of carotene.

EXPERIMENTAL

VIOLA TRICOLOR: Yellow and "apricot" varieties of pansy were grown outside in Berkeley. Three separate sets of analyses were made at different times, with slight variations in the total. For each analysis a 10 gm sample of petals was collected, extracted with acetone in a Waring blendor until colorless, and the pigment transferred to petroleum ether. A quick cold saponification of the extract with alcoholic potassium hydroxide removed traces of chlorophyll. The petroleum ether extract was washed with water, dried over anhydrous Na_2SO_4, and its spectral absorption curve obtained from measurements on the Beckman DU spectrophotometer.

The spectrum of the yellow *Viola* extract was 3-banded (fig 1), with maxima at 465, 438, and 415 mμ. Chromatography of the extract revealed a number of

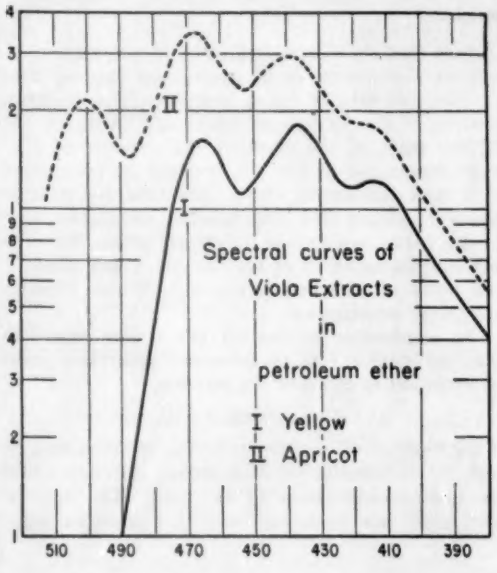

FIG. 1. Comparison of absorption spectra of extracts of *Viola* petals. Abscissa: wave length in mμ; ordinate: optical density in arbitrary units.

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xanthophylls but no lycopene. Most of the xanthophylls reacted with concentrated hydrochloric acid to give a blue color if saponified, or, without saponification, if first treated with iodine (7). There was negligible β-carotene and phytofluene. Total xanthophyll was estimated by assuming a specific absorption coefficient of 230 at 465 mμ. The error introduced by this simplifying assumption is of the order of 15 %, and is certainly less than the cumulative error in estimating at least 5 components individually after chromatographic separation.

The β-carotene and phytofluene were separated on MgO-SiO_2 columns and the volumes of the eluates measured. Concentrations were estimated by use of the coefficients 250 at 450 mμ for β-carotene and 150 at 368 mμ for phytofluene. The units are in liters/gm-cm.

The spectrum of the apricot *Viola* extract (fig 1) is characteristically that of lycopene modified by admixture of the xanthophylls. The two components were estimated by simultaneous equations based on coefficients, for lycopene, of 320 at 500 mμ and of 345 at 465 mμ, and, for the xanthophylls, of 4 at 500 mμ and of 230 at 465 mμ. Beta-carotene and phytofluene were determined as before. Results are shown in table I.

TABLE I *

COMPOSITION OF CAROTENOID MIXTURE FROM PETALS OF VIOLA TRICOLOR

VARIETY	APRICOT	YELLOW
	MG/GM DRY WT.	
Lycopene	0.39	0
Combined xanthophylls	0.33	1.25
Beta-carotene	0.004	0.004
Phytofluene	0.009	0.002

* For details, see Experimental Section.

IONONE AND THE LIGHT EFFECT: The procedure used to culture *Phycomyces* from a spore inoculum has already been described (9). By using yeast autolysate, supplemented with thiamine, we ensure rapid growth, and control cultures will produce 100 mg dry mycelium per plate in 80 to 100 hours from the time of inoculation.

Cultures were grown as follows: in complete darkness, with illumination for 4 to 6 hours after germination (i.e., 16 to 22 hours after inoculation), and in daylight in the laboratory. Each of these three conditions was further compounded by treating half the cultures with β-ionone. Cultures were then harvested, dry weights determined after lyophilization, and the carotene extracted and estimated as before (9). Results are shown in table II.

THE PREFERENTIAL SELECTION OF NON-SUGAR CARBON BY PHYCOMYCES FOR CAROTENE: Cultures of *Phycomyces* were grown as before, except that uniformly-labeled fructose was incorporated into the glucose-yeast autolysate medium. We found in a preliminary experiment that fructose could be substituted for glucose without affecting growth or carotene formation, with and without ionone.

TABLE II

THE EFFECT OF IONONE AND LIGHT ON CAROTENE PRODUCTION IN PHYCOMYCES *

	PIGMENT μG/5 CULTURES	μG/GM DRY MYCELIUM	RUN NO.
Dark:			
Control	32.1	82.8	50
	92.9	208	47
	87.2	189	61
	50	123	63
Ionone-treated	214	577	50a
	223	763	50
	245	578	47a
	564	1662	47
	171	396	61a
	139	308	61a
	186	468	63a
Light:			
1. Limited			
Control	51	150	50
	116	270	47
	110	233	61
	59	134	63
Ionone-treated	148	855	50a
	470	2090	50
	279	676	47a
	569	1576	47
	188	458	61a
	150	351	61a
	237	590	63
2. Daylight			
Control	65.4	188	47
	47.2	122	63
Ionone-treated	260	879	50
	340	781	61a
	384	860	61a
	144	442	63a

* Runs 47, 50, 61, and 63 are directly comparable within themselves. Harvest times varied from 74 to 90 hours. Ionone was added at time of inoculation, except for runs designated "a," when it was added 38 to 42 hours after inoculation. Light (limited) treatments were as follows: No. 50—6 hr, No. 47, 61, and 63—4 hr after germination of the spores in the dark.

Cultures [2] were grown on this medium with and without ionone for 90 hours after germination prior to harvest, and the carotene was then extracted. The total carotene, estimated from the optical densities of the extracts, were 250 μg from 10 control plates, and 456 μg from 10 ionone-treated plates. To each extract, treated and untreated, was added 3 ml containing 1.1 mg inactive carrier carotene, and the solutions were concentrated in vacuo to ca 2 ml, when 2 ml ethanol were added, and the vacuum concentration continued. The carotene crystallized and was removed by centrifugation, and washed with small quantities of hot ethanol.

[2] Germination was unfortunately delayed when room temperature fell to 16°C over a week end.

The carotene was oxidized to CO_2 in a micro-combustion train (using an unpacked combustion tube with 2 quartz sinters and a 3" roll of platinum in the furnace which was operated at 1000°C.) The CO_2 was collected in CO_2-free sodium hydroxide and precipitated as $BaCO_3$ by the addition of $BaCl_2$. The excess NaOH was neutralized, the precipitate was washed with water, and a known weight plated upon a 1" copper planchet. The samples were counted to a statistical accuracy of $\pm 2\%$ with a Tracerlab Auto-Scalar, using a thin-windowed Geiger tube. The activities were then corrected for self-absorption with the following results:

The medium had 406 counts/mg $BaCO_3 \times$ min.

Carotene was crystallized from both control and ionone-treated cultures.

The control carotene had 172 counts/mg $BaCO_3 \times$ min.

The ionone-carotene had 176 counts/mg $BaCO_3 \times$ min.

From this, it is clear that the ionone did not affect the specific activity of the carotene.

The dry matter of the medium was composed of 10 gm fructose, 1.2 gm yeast autolysate and 4 mg thiamine (which may be ignored in the calculations). If we assume the yeast extract to contain 40 % carbon (C), and if we further assume the carotene to be derived solely from the fructose, then the specific activity of our carotene should be:

455 counts/mg $BaCO_3 \times$ min.

In fact, it is 174 (average), i.e., $174/455 \times 100$ or 38.2 %. This means that 61 % of the C is selectively derived from the non-sugar fraction which contained only 9 % of the total C available.

This finding may be considered in conjunction with that of Grob et al (3). These authors reported data with labeled acetate. They found that 50 % of the total C of carotene was derivable from the carboxyl and 25 % from the methylene groups.

Discussion

Analyses of yellow and "apricot" varieties of *Viola tricolor* indicate that xanthophyll components are the major constituents of the yellow, while lycopene predominates in the "apricot" variety. Whereas the grapefruit involves a sequence of mutations involving changes in β-carotene and lycopene, the *Viola* mutation involves lycopene and xanthophylls. These cases do not in themselves offer conclusive evidence against a sequential pattern of carotenoid syntheses. In the absence of strong evidence to the contrary, however, they favor the parallel pathway, in which many (not necessarily all) of the carotenoids are formed individually from a common pool.

The ionone-type of approach necessarily suffers from one inherent weakness, that similar effects may ultimately be demonstrated by a series of unrelated, but as yet untested, chemical compounds. In this connection it is therefore noteworthy that an ionone effect can be shown independent of light.

Experiments on the effect of ionone (table II) indicate no significant difference between continuous and limited light. We have therefore summated and averaged the results of table II as follows:

Controls, dark, 602/4 or 151 μg per gm
Controls, light 1097/6 or 183 μg per gm
Ionone, dark 4752/7 or 678 μg per gm
Ionone, light 9558/11 or 869 μg per gm

The ratios for ionone-treated to control are 4.5 (dark) and 4.75 (light). The ratios for lighted to dark are 1.28 (ionone-treated) and 1.21 (control). This indicates that the effects are additive. We may eliminate runs designated "a" in table II, since addition of ionone, after growth has started, invariably lessens its effect on the yield of carotene. This gives a ratio, for lighted to dark of 1.17 (ionone-treated) vs 1.21 (control), which does not affect the general conclusion.

The experiment with labeled sugar indicates that in *Phycomyces* relatively little of the carotenoid C is normally drawn from the glycolytic cycle, and preferentially it is drawn from non-sugar sources. The similarity in activities of the carotenes from cultures with and without ionone is added proof that the ionone effect is an indirect one, and that the mechanism will be hard to unravel.

Summary and Conclusions

An additional source material, the petals of yellow and "apricot" varieties of *Viola tricolor*, has been analyzed for carotenoid pigments.

The ionone-effect on *Phycomyces* surpasses the effect of light, though the two are complementary.

More than 60 % of the carotene C is selectively drawn from the non-sugar fraction which comprised 9 % of the total available C. As this result was obtained with both control and ionone-treated cultures, this constitutes confirmatory evidence that the ionone effect is an indirect one.

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CAUSES OF INJURY TO FLOODED TOBACCO PLANTS[1,2]

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Tobacco is one of the most susceptible of crop plants to injury from flooding or saturation of the soil with water. It really exhibits two types of reaction to flooding, immediate but temporary wilting accompanying temporary flooding, and severe permanent injury caused by longer periods of flooding.

If the soil is suddenly saturated by a downpour of rain and the sun later shines bright and hot, sudden wilting of the leaves, often termed "flopping" by tobacco growers, sometimes occurs. This sudden wilting occurs where drainage is slow and the soil remains saturated for at least a few hours ofter a rain. High air temperatures and bright sun accentuate this sudden wilting and its occurrence may depend also on the condition of the plants, apparently being much more severe if the tobacco has been growing rapidly and therefore is somewhat soft and succulent. If the excess soil moisture drains away within a few hours the plants usually recover from this type of wilting with little or no permanent injury.

Where because of topography or an impermeable subsoil the soil in the root zone remains saturated for several days serious permanent injury or death usually results. The leaves of such plants turn yellow and die, beginning with the lower ones and progressing up the stem. Such injury is most common and severe on clay soils because they drain more slowly than sandy soils, but injury sometimes occurs even on sandy soil if poorly drained.

Considerable discussion has occurred among tobacco workers concerning the causes of the sudden wilting which often occurs immediately after a rain, but apparently little or no research has been done. Of course, the immediate effect of saturating the soil with water is to displace the air which normally occupies the noncapillary pore space of the soil. This causes a deficiency of oxygen and an accumulation of carbon dioxide, either of which might injure or kill the roots. Damage to the root systems has been assumed to result in reduction of water intake and injury to the tops from desiccation. This explanation seems inadequate, however, because wilting often occurs within an hour or two after flooding, too soon for serious injury to the roots. Furthermore, as Kramer (8) pointed out earlier, some of the symptoms of flooding injury are different from those expected in plants dying from desiccation. Wilting is often most severe in the middle leaves and in cloudy weather leaves of flooded plants often turn yellow and die with little or no wilting. Epinasty also is common, though the change in angle of curvature is so small in tobacco that it is generally overlooked.

These observations suggested the need for a careful study both of the visible symptoms of flooding injury and of the physiological causes of these symptoms.

THE CAUSE OF SUDDEN WILTING

Previous work (7) indicated that either an O_2 deficiency or an excess of CO_2 greatly reduced the permeability of the roots of sunflower and tomato to water, and Hopkins, Specht, and Hendricks (5) reported wilting of tobacco growing in sand cultures deficient in O_2. An experiment was therefore performed to measure the effect of flooding on the permeability of tobacco roots to water.

A group of tobacco plants (variety Dixie Bright 101) was grown to the height of about 25 cm in 4-inch pots filled with sandy loam soil. The pots were submerged in a tank of water and at intervals six plants were removed for permeability tests. The tops were cut off about 3.5 cm above the soil surface and 5-ml graduated pipettes with cut off tips were attached to the stumps by means of rubber tubing. The joints were sealed airtight with melted grafting wax and enough water was added to each pipette to bring the meniscus into the graduated region. The pipettes were attached to a vacuum line and the root systems subjected to a pressure gradient of 0.2 atmosphere. The rate of water intake was recorded for 15 minute intervals and the results are shown graphically in figure 1 and part of them are also given in table I. The rate of water intake after only an hour of flooding fell to 60 % of the rate after 15 minutes of flooding. At hour intervals new groups of flooded root systems were detopped and attached to the vacuum line and they showed a steady decrease in rate of water intake until the rate levelled off after three hours of flooding at about 25 % of the rate after 15 minutes of flooding. This experiment shows clearly that flooding the soil

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[2] This research was supported in part by grants from the Research Council of Duke University.

TABLE I

WATER INTAKE IN ML PER HOUR PER ROOT SYSTEM FOR 9 DIFFERENT GROUPS OF TOBACCO PLANTS TREATED AS INDICATED AND THEN SUBJECTED TO A PRESSURE GRADIENT OF 0.2 ATM.*

PLANT NO.	SOIL FLOODED					SOIL SATURATED		SOIL FLOODED	
	15 MIN	3 HR	24 HR	4 DAYS UNAERATED	4 DAYS AERATED	4 DAYS CO_2	4 DAYS N_2	6 DAYS 20°C	6 DAYS 34°C
1	0.24	0.12	0.65	...	0.47	0.12	1.63	0.00	...
2	0.72	0.13	1.09	3.48	0.24	0.00	0.12	0.23	...
3	0.60	0.17	0.32	0.13	0.17	...	0.08	0.05	...
4	0.60	0.11	0.84	0.58	...	0.05	0.19	0.11	2.89
5	0.80	0.19	0.95	4.66	0.71	0.00	0.18	0.17	...
6	0.80	0.19	0.72	...	1.09	0.13	0.17	0.03	1.72
Average	0.63	0.20	0.76	...	...	...	...	0.10	...

* Water intake cited in ml per hour per root system. Soil temperatures fluctuated from 25 to 35°C except in the last two columns.

results in a rapid decrease in permeability to water which causes the sudden wilting or "flopping" often observed in the field.

The subsequent behavior of the root systems also is interesting. After 24 hours the rate of water intake had risen again and was higher than after 15 minutes of flooding, and it was nearly five times as high after 43 hours. These results suggest that the roots were sufficiently injured after a day or two to increase their permeability, but not damaged enough to decrease their efficiency as absorbing surfaces or conducting structures. The results of this experiment are similar to those obtained by Hoagland and Broyer (4) with a tomato root system in nutrient solution

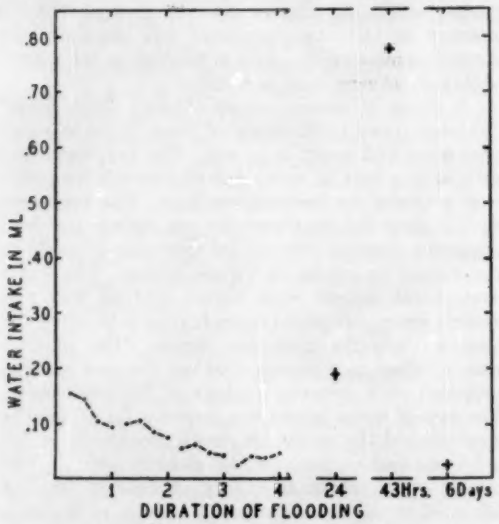

FIG. 1. The effect of flooding for various periods of time on water intake through tobacco root systems attached to a vacuum line and subjected to a pressure gradient of 0.2 atmosphere. Each point is the average water intake through six root systems for a 15 minute period at 25 to 30°C.

saturated with CO_2. In their experiment water intake dropped to about 20 % of the original rate within an hour after the roots were exposed to CO_2, then rose after 18 hours to more than twice the original rate.

Water intake through roots flooded more than two days was quite variable, as can be seen in table I. In an earlier experiment (8) practically no water intake occurred under pressure through root systems flooded four days, but in these experiments roots flooded four or more days showed so much individual variation that average values have little meaning. This individual variation within experiments is believed to result from differences in rate of decay of the injured root systems. The high rates of water movement recorded in table I for some root systems probably occur because decay has softened some of the tissue until it collapses under pressure and allows mass flow of water. The usual course of events probably is as follows. Deficient aeration produces an immediate but temporary decrease in permeability, possibly caused by increased viscosity of the protoplasm. Continued deficient aeration results in injury and finally death of at least some cells, resulting in an increase in permeability. Plugging of the xylem vessels begins to occur, decreasing permeability, but decay finally results in an increase in permeability or in complete destruction of the root system.

FACTORS AFFECTING SEVERITY OF INJURY TO FLOODED PLANTS

If the wilting accompanying saturation of the soil with water is caused by inadequate aeration, then aeration of saturated soil ought to prevent wilting and injury, and displacement of the air in unflooded soil with CO_2 or N_2 ought to cause wilting and injury similar to that caused by displacement of the air with water. An experiment was therefore performed to test these possibilities. The various treatments used are shown in table II. The tobacco plants used were about 25 cm in height and were growing in 4-inch pots of sandy loam soil. There were six plants in each treatment. The root systems and accompanying soil masses were carefully transferred to metal

cans, except in treatments 5 and 6 where the soil was washed from the roots which were then supported in coarse gravel in the cans. This was done to learn whether or not injury would be as great in the absence of soil as in its presence. The cans used in the experiment were provided with inlet tubes at the bottom for entrance of gas. The treatments were started in midmorning and were continued for four days. The results are summarized in table II and are in accord with our original hypothesis.

Aeration of flooded soil by forcing air up through the bottom of the can prevented wilting and greatly decreased the severity of injury from flooding to both roots and shoots. Displacement of the air in unflooded soil at field capacity by CO_2 produced the most rapid and severe wilting the first two days, but the plants showed little wilting the third and fourth days, although they showed severe chlorosis and general injury. Displacement of the soil air with N_2 caused less rapid and less severe wilting than displacement with CO_2. Removal of soil from around the roots did not decrease injury from flooding to any noticeable extent, but aeration decreased injury to the plants with soil-free root systems about as much as it decreased injury to plants rooted in soil.

Attempts to measure the permeability of the roots to water at the end of this experiment were not very successful because many of the stems were badly decayed and collapsed when vacuum was applied. Apparently some roots were so decayed that water passed through very readily, while in others the conducting system in the bases of the stems was plugged, allowing very little passage of water. There was no relation between the condition of the shoots and the permeability of the roots within a given treatment, suggesting that inability to absorb and translocate sufficient water is not the only cause of injury to the shoot.

Soil temperature might be expected to affect the speed and severity of injury to flooded plants. This was tested by immersing six potted tobacco plants in a water bath maintained at 20°C and six similar plants in another water bath maintained at 34°C. The experiment was started at noon and the plants in the cooler tank wilted slightly within an hour, but those in warm water did not wilt for about 3 hours. The following day the plants in warm water were much more severely wilted and remained more severely wilted during the remainder of the experiment. Chlorosis was also much more severe in the plants at the higher temperature and by the fourth day the submerged portions of their stems had begun to decay and were turning brown and the shoots showed much more injury than those at 20°C. Shoot growth ceased within 24 hours after flooding in the warm tank while the plants in the cool tank continued to grow at the same rate during the entire six days of flooding. At the end of the experiment the root systems of plants in the warm tank were so badly decayed that it was impossible to measure their permeability accurately. In contrast the root systems kept at 20°C showed only limited decay, but showed very low permeability to water. Although the bases of the stems on these root systems appeared healthy the vascular bundles

TABLE II

EFFECTS OF VARIOUS FLOODING AND GAS TREATMENTS ON TOBACCO PLANTS *

TREATMENT	REACTION ON FIRST DAY OF FLOODING	REACTION ON SECOND DAY OF FLOODING	REACTION AFTER FOUR DAYS OF FLOODING
1. Soil flooded without aeration	Severely wilted at midday. Plants flooded 1 day and drained recovered.	Slightly wilted at midday. Plants flooded 2 days and drained did not recover.	Severely wilted early in morning. Severe chlorosis of leaves.
2. Soil flooded with aeration	No wilting.	No wilting.	No wilting at any time. Very slight chlorosis.
3. Soil at field capacity, saturated with CO_2	Severely wilted within an hour. Recovery in late afternoon.	Wilted earlier and more severely than plants with other treatments.	Unwilted early in morning and only slight wilting at midday. Moderate chlorosis.
4. Soil at field capacity, saturated with N_2	Moderately wilted at midday, recovered in late afternoon.	Moderately wilted by noon.	Lower leaves slightly wilted early in morning, only slight wilting at noon. Moderate chlorosis.
5. Roots washed free of soil and flooded without aeration	Less severe wilting than in plants with roots in soil (Tr. 1).	Upper leaves severely wilted by noon and all leaves wilted in afternoon.	Severe wilting early in morning. Some death of leaf tissue. Severe chlorosis of leaves.
6. Roots washed free of soil and flooded with aeration	No wilting.	Slight wilting at midday.	Slight wilting at midday. Slight chlorosis.

* Six plants per treatment.

were discolored. The low rate of water intake through these roots probably was caused by plugging of the xylem. Such plugging is a common occurrence where tissue is injured in any way (6).

It should be noted that the water in which these pots were immersed was stirred continuously, hence was much better aerated than it is in flooded soil. Nevertheless injury was almost as severe as in plants flooded in quietly standing water. The results of this experiment leave no doubt that soil temperature affects the rate and extent of injury to plants.

Discussion

The results of these experiments show that the sudden wilting or "flopping" which often occurs within a few hours after the soil is flooded results from a temporary decrease in permeability of the roots to water. The presence of an excess of water is not itself the cause of decreased permeability because wilting and injury were prevented when flooded root systems were well aerated. Some question exists concerning the relative importance of O_2 deficiency and CO_2 excess. Saturation of the soil with CO_2 displaces the O_2 (and incidentally the N_2) while saturation with N_2 displaces both O_2 and CO_2. Plants wilted sooner when the soil air was displaced with CO_2 than when it was displaced with N_2, suggesting that an excess of CO_2 has more immediate effects on permeability than a deficiency of O_2. Earlier experiments in this laboratory showed that saturation of the soil or water surrounding the roots of sunflowers and tomatoes with CO_2 decreased water absorption 35 to 50 % within an hour, while displacement of O_2 with N_2 caused a decrease of only 10 % (7). Chang and Loomis (2) found water absorption by corn, wheat, and rice to be reduced 15 to 50 % by bubbling CO_2 through solutions in which the plants were growing for 10 minutes of each hour. Apparently CO_2 has a specific effect on protoplasm, causing increase in viscosity and decrease in permeability (3, 9).

High soil temperature would be expected to increase the rate and severity of flooding injury because it can act in several ways. Root respiration increases with increasing temperature, causing an increase in O_2 requirement which probably results in more rapid injury than would occur with a low O_2 requirement. Cannon (7) found that a higher O_2 concentration was required for root growth at high temperatures than at low temperatures, and the same relation probably holds for survival of roots. High temperatures also increase the activities of microorganisms, speeding up the depletion of O_2 and the accumulation of CO_2. Perhaps equally important is the increased severity of injury to the root systems and bases of stems of flooded plants. At 20°C the root systems showed little damage, but after a few days at 34°C the roots and stem bases were dead and decaying. In addition to the destruction of absorbing surfaces, it is possible that toxic substances were carried upward and contributed to the injury of the shoot. In view of these possibilities it is not surprising that injury from flooding is less marked at lower temperatures. At least one instance is known where young tobacco plants in a seed bed were flooded by a stream for several days in the early spring without injury. Unfortunately most flooding in the fields occurs when soil temperatures are high enough to intensify the injury.

The role of soil microorganisms in contributing to flooding injury deserves more study. Flooding causes a shift in the population of soil organisms toward anaerobic forms, some of which produce reduced substances such as nitrites and sulfides that are highly toxic to roots. Tobacco roots already injured by O_2 deficiency seem very susceptible to attack by microorganisms. In a previous study (8) it was found that tobacco plants growing in pots of sand were injured less by flooding than those growing in pots of soil, possibly because there were fewer organisms in the sand. In the present experiments washing the roots free of soil before immersion in water did not reduce injury from flooding. Unpublished data obtained by the junior author in another series of experiments indicated that removal of soil from around the roots often decreases injury from flooding. Removal of soil may somewhat increase gas exchange between the roots and the surrounding medium, and it also removes most of the soil organisms from the roots.

The duration of flooding, as would be expected, has a strong bearing on the severity of injury. Plants flooded for only one day usually recovered with little or no permanent injury, but plants flooded two or more days showed permanent injury and plants flooded five or six days were injured beyond recovery.

The relation between aeration experiments and flooding injury deserves mention. Hopkins, Specht, and Hendricks (5) found considerable growth of tomato and tobacco roots in an atmosphere containing 1.5 % of O_2 and the shoots made some growth in an atmosphere containing only 0.5 % O_2, although the roots decreased in weight in the latter atmosphere. Their gas mixtures were circulated through the sand in which the plants were growing at a rate of 10 liters per hour, removing CO_2 and producing conditions very different from those existing in a moist or saturated soil where mass movement of gas is impossible. In a heavy soil, even at field capacity, diffusion of gas is so slow that a serious O_2 deficiency may develop around individual roots or groups of roots at much higher average O_2 concentrations than are required where the O_2 is forced through the soil. In flooded soil gas movement is even more limited, hence O_2 deficiency and accumulation of CO_2 occur even more rapidly. Although flooding injury is caused by poor aeration it is more complex in origin than injury caused by simple O_2 deficiency and probably cannot be simulated by aeration with gas mixtures low in O_2 concentration.

Summary

Tobacco shows two types of reaction to flooding of the soil. Severe, but temporary, wilting often occurs within an hour or two after the soil is saturated

if transpiration is rapid, but the plants recover if the soil drains promptly. If the soil remains saturated more than 24 hours permanent injury occurs. Stem elongation usually ceases, epinasty often occurs, the leaves turn yellow and die, beginning at the base and progressing up the stem, and the roots and base of the stem begin to die and decay. Injury develops more rapidly in warm than in cool soil.

The initial wilting is caused by a sudden decrease in permeability of the roots to water. Prolonged flooding causes injury and death of the roots because of deficient aeration, but this is aggravated by the activity of microorganisms which destroy the roots and plug the xylem in the base of the stem. It also is possible that toxic substances produced by microorganisms or in the dying root cells contribute to injury of the shoots. The role of microorganisms in flooding injury deserves further investigation.

Wilting and injury to flooded plants can be prevented by forced aeration of the soil, and plants growing in soil at field capacity can be caused to wilt and show symptoms of injury by displacing the soil atmosphere with CO_2 or N_2. Use of CO_2 produces wilting sooner than N_2.

Although injury to plants in flooded soil is caused by deficient aeration it is more complex in nature than injury from O_2 deficiency alone because in most instances microorganisms appear to be involved.

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SUBCOOLING AND ICE NUCLEATION IN LEMONS[1]

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Subcooling of living tissue has been the subject of periodic study for several decades. The characteristic of plant tissue, in particular, to cool somewhat below its freezing point before ice begins forming in it may be of practical importance because, as is well known, subcooling in itself does not cause freezing damage (3, 4, 23, 24, 29). Several reviews concerning tissue subcooling have appeared, although most are only portions of publications dealing with low temperature damage in general (16, 18, 34, 39). Müller-Thurgau (23, 24) investigated subcooling by inserting a mercury thermometer into the plant part cooled and obtained the temperature curve now recognized as characteristic for subcooling with subsequent freezing. This work showed that the difference between the lowest temperature attained before freezing and the tissue freezing point, referred to as the amount or degree of subcooling in this paper, ranged up to 10°C. Later, Diehl and Wright (4) used thermocouples but otherwise similar methods and observed 4°C subcooling in apples. Single grape berries subcool 3°C on the average according to Carrick (2), and Schoonover et al (28) suggest Navel oranges are able to subcool 1.5°C in the orchard.

The theory of nucleation promises to provide a sound fundamental basis upon which to develop a study of subcooling in living tissue. This theory concerning the mechanism of initiation of the more stable phase within a metastable phase is reaching a stage in which it may be profitable to apply it to the study of subcooling in a complex system such as that of plant tissue. Several reviews dealing with nucleation theory have appeared (1, 12, 14, 38). The fundamental assertion of this theory is that the stability of a metastable phase lies in the existence of an activated complex or nucleus stage through which at least one group of molecules must pass before the more stable phase

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can grow. The energy of activation for nucleation, W, is due to the relatively large contribution made by the interfacial energy to the average energy per molecule in the nucleus. Ice nuclei in subcooled water, for instance, may contain on the order of only 80 molecules (40).

Volmer and Weber (35) employed the fluctuation theory of Einstein (7) to obtain the general expression for the rate of nucleus formation, I, per unit volume of homogeneous or pure metastable phase:

$$I = Ke^{-(W/kT)} \quad (1)$$

where K is a constant later evaluated by Farkas (8) for liquid nucleation in a supersaturated vapor and by Turnbull and Fisher (31) for crystal nucleation in a subcooled liquid, k is Boltzmann's constant, T is the absolute temperature, and W is calculable by a formula derived by Gibbs (9). This equation accurately predicts the experimentally measured rate of liquid nucleation in supersaturated vapors free of dust and ions (37). Turnbull's (32) determinations of the rate of crystal nucleation in those samples of sequestered mercury droplets which subcooled most (homogeneous nucleation apparently took place in these) are also in good agreement with equation (1).

The catalyzing action of various particles and of spots on solid surfaces upon nucleation has received attention (12, 36). Such surfaces evidently reduce the energy of nucleation activation and in this way cause heterogeneous nucleation. Volmer (36) proposed adapting equation (1) to account for this effect by multiplying W by a factor containing the equilibrium contact angle between the stable phase and catalyst surface in the presence of the metastable phase. Using this suggestion Turnbull (32) found excellent correspondence with the rate of crystal nucleation in groups of mercury droplets which subcooled less than the maximum. Particles which catalyze ice nucleation in subcooled water are known as freezing nuclei by meteorologists.

As the rate of nucleation is an exponential function of the temperature, it is extremely temperature sensitive. This temperature sensitivity is exemplified in the experimental findings that homogeneous crystal nucleation in subcooled mercury occurs in a temperature range measured in tenths of a degree (32), and that heterogeneous ice nucleation due to a particular freezing nucleus repeatedly occurs within a few tenths of a degree of the same temperature (5, 40). It is clear that nucleation is a probability occurrence only in so far as the chance of there being present a foreign substance capable of causing heterogeneous nucleation under the ambient conditions is concerned.

Apparatus and Procedure

With the exception of some vesicles which were held in a walk-in freezer for microscopic examination, all tree parts were cooled in a closed wooden container, 30 cm on a side. Fruits were supported by their stems, which were clamped in alligator clips lined with Scotch electrical tape. In several runs the entire cut stems were maintained above 0°C by inserting them into heating caps made of spiraled nichrome wire and covered with a 0.5 cm layer of asbestos (fig 1). The specimen container was cooled in a compartment of a household reach-in freezer while the temperature between it and the compartment walls was maintained uniform with a small fan. A temperature fluctuation of ± 1°C outside the container assumed negligible proportions inside it, as its walls contained a 1.5 cm thickness of glass wool. The thermostat was controlled by clockwork so that the rate of temperature decrease was a constant 1.1°C per hour. This rate compared favorably with that in the orchard on winter nights and also provided for a reasonably uniform temperature within the specimens during cooling.

Fig. 1. Lemon in specimen container with thermocouple and stem heater in place.

Temperature measurement within the container was by means of iron-constantan thermocouples of 30 gauge wire. Those employed inside tissue were coated with Glyptal. In several series of runs, temperatures were measured with thermocouples whose uncoated wires were wound in flat spirals around the junctions as shown in figure 1. Each junction was placed 1 mm above the surface of the fruit. The purpose of the spiral shape was to permit the lengths of thermocouple wire which could affect the temperature of the junction to be approximately the same distance from the fruit surface as the junction itself, and also to allow reproducible results. Temperatures were recorded by a twelve-point recording potentiometer (Leeds and Northrup, Micromax Model) with a range of −18 to 93°C. The error of temperature measurement was ± 0.09°C, as determined by the standard

deviation from 0°C of a reference distilled water-ice mixture. This relatively small error was obtained by taking certain precautions: (a) thermocouple wire from the same spools of iron and constantan, respectively, was used throughout; (b) the potentiometer was calibrated with an ice bath after alternate runs; (c) dead space error was eliminated by causing the recorder to approach all temperatures of interest from the same direction (all odd-numbered points indicated the temperature of the relatively cold inside wall of the freezer compartment); (d) the error of reading the recorded temperatures was minimized by measuring the position of the points on the chart paper with a microscope equipped with an ocular scale. An additional uncertainty of $\pm$ 0.05°C was due to the circumstance that the temperature of each thermocouple was recorded at six-minute intervals.

Humidity was controlled by sulfuric acid solutions, and a lining of polyethylene film on the inside walls of the container served as a vapor barrier. Checks of actual humidities were made by the dew point method. The start of dew or frost formation on the surface of a copper bar projecting inside was detected visually while the temperature fall of the area observed was followed. The relative humidities read from appropriate psychrometric charts corresponded with those given for the various concentrations of acid used.

All specimens were obtained during the spring from a Eureka lemon tree grafted on grapefruit root. The tree was untreated with either an insecticide or a fungicide, and it received no special treatment during the course of the experiments. Lemons commercially graded as silver were used exclusively; their diameters varied from 41 to 54 mm.

The procedure for measuring the ice nucleation point, or the temperature at which freezing began, was unvaried throughout. Fresh specimens were placed in the container, which was then kept in a walk-in freezer held at 4 $\pm$ 1°C for approximately ten hours before being transferred to the reach-in freezer compartment. For the determination of the nucleation point of individual fruits, groups of nine were cooled three at a time. Preliminary runs established that, during cooling, the spiral thermocouples registered 0.42 $\pm$ 0.16°C less than thermocouples in the fruit centers, and 0.05 $\pm$ 0.11°C less than thermocouples imbedded 1.5 mm under the fruit surfaces and the same distance around the fruits from the stems as the junctions of the spiral thermocouples. The temperature of each spiral thermocouple clearly indicated the onset of freezing in the fruit over which it was held, as the readings of the thermocouple and point fruit temperatures, taken at one-minute intervals, were found to rise together.

Results, Analysis, and Discussion

Subcooling in Vesicles and Spread of Freezing: Individual detached vesicles, 85 in number and resting on microscope slides, were placed in three closed Petri dishes in the wooden specimen container and the temperature lowered. The temperature was measured by means of a thermocouple inside an extra vesicle in one of the dishes. At −12°C no vesicle had begun freezing, but when the temperature had fallen below −18°C, 42 vesicles were frozen. A thermocouple whose temperature was about −12°C was thrust into several of the subcooled vesicles, one at a time. In each case, the vesicle froze very rapidly, and the temperature recorded by the thermocouple rose to approximately −2°C, showing that the low temperature attained without freezing was not due to a lowering of the freezing point in the detached vesicles.

Two sets of seven detached vesicles were placed so that the cut stalk ends were in separate pans of water while the bulbous portions remained in air. The pans and vesicles were placed in a −7°C atmosphere. In a few hours the water in one pan and all the surrounding vesicles were frozen. In the other pan neither the water nor any of the vesicles was frozen after 24 hours. At that time the water was inoculated with ice; it and all the vesicles around it quickly froze. This result indicates that freezing readily spreads through the stalks. As closer study of this process seemed desirable, several detached vesicles on microscope slides were placed in a walk-in freezer maintained at −8 $\pm$ 2°C and observed through a low-power stereoscopic microscope a few hours later. Within a few seconds after ice was pressed against the cut stalk ends very narrow, threadlike columns of ice penetrated to the point where xylem vessels terminate. The subsequent freezing in the bulbous portions of the vesicles was intermittent and quite clearly intracellular. The velocity with which the cellular freezing front progressed in the vesicles was several orders of magnitude less than the crystallization velocity in the stalks. These results suggest that rapid freezing spreads in stalks and throughout the vascular system of a fruit by way of the water conducting vessels.

It was found that vesicles subcooled to −7°C were not inoculated by ice held against their sides. To further investigate the mode of spread of freezing in the pulp, separate segments were cooled on glass slides. The segment membrane on each side was pulled back exposing the vesicles and two thermocouples were inserted into vesicles at opposite ends of the segments. When the indicated temperature reached −5°C, the vesicles punctured by one of the thermocouples were inoculated. In less than one minute the freezing spread to the vesicles around the thermocouple at the opposite end, as indicated by a temperature rise there similar to that in the inoculated portion. However, in segments from which the peel, including the inner wall of the outer portion of the segment and the vascular bundles connecting the vesicle stalks, had also been removed, the freezing did not penetrate to the vicinity of the other thermocouple in 10 minutes. It follows that freezing is disseminated throughout a segment by way of the vascular system and does not progress directly from one vesicle to another inside the segment.

A rough check on the rate of freezing spread in a subcooled system comprising a fruit and stem with attached leaves was made. The stem was held horizontally in the container, and the temperatures at four points were recorded: one thermocouple was inserted 0.5 cm into the cut stem end; another was imbedded in the midrib of a leaf; the third was inside the pulp of the fruit; and the last was 2.5 cm below the stem, midway between the fruit and the cut stem end. Several runs were made, and subcooling was followed each time by apparently simultaneous freezing throughout the system, as indicated by the fact that all the temperatures, taken at two-minute intervals, rose together. The recorded air temperature increased least so that air heated by the fruit, for instance, could not have caused the temperature in the leaf or stem to increase the recorded amount without freezing actually occurring there; the stem was placed in a horizontal position to reduce this possibility to a minimum. Freezing was found to spread from one fruit to another on the same branchlet in a similar manner. The crystallization velocity was found to be greater than 15 cm per minute at a subcooled temperature of −5.2°C. It is expected that a more precise measurement would show the crystallization velocity to be considerably above the lower limit established.

As xylem vessels are continuous tube-like structures containing a dilute aqueous solution, it is reasonable to expect the crystallization velocity in them to compare with that found in glass capillary tubes. Tammann and Büchner (30) give a value of 66.5 cm per minute in water subcooled 3.2°C, which is well above the lower limit obtained for branchlets. That freezing spreads rapidly in subcooled plant tissue was suggested by Müller-Thurgau (24), who also obtained evidence that freezing spreads through water-conducting vessels in potatoes. Maurer and Murray (20) noted that ice first forms in and around these vessels. The finding by Seemann (29) that freezing spreads almost instantaneously in subcooled bean plants is similar to the result reported here. The fact that freezing spreads rapidly in subcooled tissue strongly suggests that ice nucleation ordinarily occurs at one point only in such a system, just as it does in small volumes of subcooled water.

A Thermodynamic Analysis: The results given above are used together with concepts of the theory of nucleation and the supporting experimental results to carry out a thermodynamic analysis of the phenomenon of ice initiation in a lemon. The system examined is composed of a fruit, its stem, and the surrounding atmosphere. It is postulated that ice nucleation occurs neither in the stem nor in the atmosphere. The tissue is idealized to consist entirely of healthy undamaged cells, and its interior is postulated to be in equilibrium with respect to water; that is, that the partial molal free energy with respect to water is uniform there. This does not mean, as Edlefsen and Anderson (6) have emphasized, that the freezing point is uniform. On the contrary, as proteins and other substances are concentrated at protoplasmic interfaces (15), a lower freezing point at those interfaces is to be expected. Such a decrease in freezing point is evidenced, for example, by the action of the outer protoplasmic membrane in inhibiting ice growth through it (3). For this reason, ice nucleation is not likely in the outer protoplasmic membrane, and the fruit is seen to be divided into two distinct parts in so far as ice nucleation is concerned: (1) the intercellular portion, and (2) the intracellular portion.

A very important finding of nucleation studies is that homogeneous ice nucleation does not take place above −39°C in subcooled water (27). This implies that the presence of freezing nuclei active at small degrees of subcooling is a prerequisite for ice nucleation at temperatures experienced in the orchard. The finding that vesicles subcool below −12°C, together with the fact that the location of initial ice formation has consistently been found to be in the intercellular spaces (3, 17, 23, 24), suggests that ice nucleation does not take place within the pulp but, rather, in the intercellular space of the peel. Small, freezing nuclei undoubtedly can diffuse into the intercellular space, as these nuclei need be only of a size comparable to ice nuclei (13). As a consequence, the reports that soil particles (27) and atmospheric dust (25, 27) catalyze ice nucleation in water subcooled a few degrees, lead, with one exception, to the following hypothesis. Ice nucleation in a lemon ordinarily occurs at or near its surface and is due to freezing nuclei of atmospheric origin. The exception is based upon the evidence of Dorsey (5) and Wylie (40) that solid surfaces rubbed together in water catalyze ice nucleation at a slight degree of subcooling. This suggests that lemons wet with dew at their point of contact and brushing against one another cannot subcool as much as other fruits. If the hypothesis is correct, varying conditions at the fruit surface should affect the degree of subcooling; it was on this basis that the experiments described below were made.

Subcooling of Detached Lemons: The freezing points of a group of detached fruits were obtained by placing thermocouples in their pulps and cooling them in the specimen container. The highest temperature maintained for 15 or more minutes, once freezing had begun, was taken as the freezing point. The amount of previous subcooling had no apparent effect on the measured freezing points. The result is given in table I.

The ice nucleation point of fruits with (fig 1) and without stem heaters in place was determined under three conditions of humidity. Temperatures were measured with spiral thermocouples, as it was desired to have no foreign body whose surface could possibly aid ice nucleation projecting into the fruits. The surfaces of all the fruits in these five groups appeared dry to the naked eye throughout the tests. Frost formed on the inside surface of the plastic lining in the 90 % relative humidity runs but had no apparent effect on fruit subcooling. There was no significant difference in nucleation point among these five groups (table I).

TABLE I

EFFECT OF SURFACE CONDITIONS ON SUBCOOLING OF DETACHED LEMONS AND LEMON PARTS

GROUP	TREATMENT	ICE NUCLEATION POINT *
		°C
1	Freezing point of fresh fruits	−1.4 ± .05
2	Detached vesicles	below −12
3	Fruits in 90% relative humidity	−4.4 ± .20
4	Fruits in 90% relative humidity and stems heated	−4.2 ± .30
5	Fruits in 55% relative humidity	−4.0 ± .25
6	Fruits in 8% relative humidity ...	−3.9 ± .25
7	Fruits in 8% relative humidity and stems heated	−3.9 ± .29
8	Condensation on fruits	−4.3 ± .15
9	Fruits in alcohol	−6.1 ± .34
10	Half peeled fruits in alcohol	−8.7 ± .40
11	Fully peeled fruits in alcohol	−8.5 ± .36
12	Fruits rubbed on water droplets ..	−2.6 ± .07
13	Fruits rubbed but not on droplets	−4.1 ± .18

* The values are the mean of 9 fruits ± standard error.

The effect of surface moisture on the degree of subcooling was determined with two groups of fruits. Moisture was formed on the fruit surfaces of group 8 by condensation from relatively moist laboratory air which was forced into the specimen container at half-degree cooling intervals. Frost deposition on the inside surface of the plastic lining was prevented by a thin coating of glycerol. The fruits in group 13, whose treatment is given in more detail below, were cooled with distilled water droplets on their surfaces. Subcooling of fruits in these two groups was not significantly different from that of fruits with dry surfaces. This is somewhat similar to the finding of Seemann (29) that spraying bean plants with water immediately before cooling appears to slightly increase subcooling.

Another group not included in table I was treated just as was group 8 except that frost was allowed to form on the plastic lining. The mean of the ice nucleation points was −2.4°C. The higher temperature was apparently due to ice inoculation of the fruits by frost particles from the lining. This result compares with those reported previously by several workers (21, 22, 28, 33, 39), and it suggests that either a similar phenomenon or rubbing, discussed below, may have affected their results.

During the course of preliminary condensation experiments, it was noted that the appearance of ice on a fruit surface was followed, a short time later, by freezing of the fruit. This finding compares with that of Harvey and Wright (11), who found that frozen water on tomatoes prevents their subcooling; of Wartenberg (39), who obtained similar results with seeds; and of Young (41), who states that citrus fruits covered with ice appear to subcool less. The heavy covering on some herbaceous plants which can prevent ice inoculation from the surface (10) is evidently not present on lemons.

Fruits completely submerged in C.P. methyl alcohol were cooled. The temperature recorded was that of a thermocouple resting on the top surface of each fruit. These fruits subcooled more than fruits cooled under both dry and wet surface conditions (table I), and the differences are significant below the 1% level as determined by the "t" test. The freezing points of sample fruits obtained with thermocouples in the pulps during a second freezing exhibited no deviation from the freezing point of fruits not previously in alcohol. This appears to indicate that the lower nucleation point of fruits in alcohol cannot be attributed to internal dehydration. Two aditional groups of fruits were cooled in alcohol. In the first, the outer half of the peel was removed before the fruits were placed in alcohol; in the other, the entire peel was removed leaving only the outer segment walls and imbedded vascular tissue. The nucleation points of these two groups, given in table I, do not significantly differ. However, the difference between their subcooling and that of unpeeled fruits in alcohol is statistically significant below the 1% level even though the lowering of freezing point in the peeled fruits is taken into account: the freezing point of half peeled fruits was about 0.5°C lower than that of other fruits, and that of fully peeled fruits was about 0.75°C lower. These results resemble those recently reported by Luyet and Gehenio (19), who found greater subcooling of tissues predipped in glycerol.

Immersion in alcohol undoubtedly greatly reduces the freezing point, and also the chance of ice nucleation, at and adjacent to the surface of the fruits. For this reason, the results indicate that ice nucleation occurred near the surface in the fruits cooled in air.

The prediction that rubbing together of wet fruit surfaces hinders subcooling was checked with group 12. When the temperature of a spiral thermocouple first dropped to −1.5°C, and at each half degree below that, fruits wet at the point of contact were brought together and rubbed against one another. These fruits subcooled less than any other fruits tested, and the difference in each case is statistically significant below the 1% level. As a check, fruits in group 13 were cooled with distilled water droplets on their surfaces and were rubbed together, but not at the location of a droplet. These fruits subcooled to an extent comparable to those cooled with no rubbing and no water droplets on them. The effectiveness of rubbing of wet fruit surfaces in reducing subcooling may explain reports that wind lessens subcooling in plants (22, 28).

The hypothesis offers explanations for many previous findings concerning subcooling of plant parts. Müller-Thurgau's (23) discovery that factors which affect degree of subcooling are not the same as those which determine freezing point in living tissue is in agreement with the concept that the kind of freezing nuclei present determines the nucleation point. That there is no relation between rate of cooling, cell size, or osmotic pressure of cell sap and amount of subcooling (34) also agrees with nucleation theory, which asserts that minimum temperature at a point determines

whether ice nucleation will occur there, all other conditions being the same. In addition, the hypothesis indicates that neither the temperature gradient nor the length of time at a given temperature determines whether or not ice nucleation will occur. It may be possible to explain the finding that subcooling ability increases as the cross-section of intercellular spaces decreases (34), if the assumption is allowed that freezing nuclei from the atmosphere penetrate more quickly and more deeply into tissue with larger intercellular spaces. The hypothesis may also lead the way toward an understanding of reports that plants subcool more as turgor decreases (22, 26, 29). Tissue under water stress may absorb sufficient moisture to lower the dew point of air near it; dew, which aids nucleation when rubbing occurs, would then form on the plant surface under higher atmospheric humidity or lower surface temperature than otherwise.

Summary

Some low temperature phenomena in lemon tissue were investigated by means of cooling apparatus constructed for the purpose.

Detached lemon vesicles were found to subcool readily to −12°C.

Experiments and observations made suggest that freezing in lemon segments progresses by way of xylem vessels.

The spread of freezing in lemon tree parts, all portions of which are subcooled, was shown to be very rapid. The crystallization velocity in branchlets has been found to be in excess of 15 cm per minute. The rapid spread of freezing suggests that ice nucleation occurs at a single point in a subcooled tree part.

A hypothesis that ice nucleation in a subcooled lemon takes place upon freezing nuclei of atmospheric origin near the fruit surface has been developed. The following results are consistent with the hypothesis.

(a) Humidity of the surrounding air or moisture on the fruit surfaces had no effect on the ice nucleation point of detached lemons.

(b) Fruits submerged in alcohol subcooled more than fruits in air.

(c) Fruits peeled before submersion in alcohol subcooled even more than unpeeled fruits in alcohol.

The additional prediction that rubbing of wet fruit surfaces may greatly hinder subcooling has been experimentally verified.

The hypothesis has been found to offer explanations of some plant subcooling phenomena observed by others.

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THE RELATIVE SENSITIVITY OF XANTHIUM LEAVES OF DIFFERENT AGES TO PHOTOPERIODIC INDUCTION[1]

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Lang (5), in his recent review, has discussed the literature dealing with the relative sensitivity to photoperiodic treatment of leaves of different ages. The work of Moskov (8, 9) with chrysanthemum, Borthwick and Parker (1) with Biloxi soybean, and Naylor (10) with Xanthium has indicated that the first expanded leaf showed the greatest sensitivity of those tested. Hamner and Bonner (3) in 1938, and others since, found that Xanthium plants defoliated except for the youngest leaves of less than 1 cm^2 area did not appear sensitive to short day treatment.

The tacit assumption has been that sensitivity to photoperiod increased as leaves expanded until the leaf reached full size and then gradually decreased as the leaf became older.

In most short day plants several photoinductive cycles are required to induce flowering. Xanthium is known to differ from these other short day plants since with this plant one short day is sufficient to bring about flower initiation. It is possible, therefore, to measure precisely the relative sensitivity of young expanding leaves of this plant. The results of certain experiments in this laboratory made it appear desirable to reexamine the photoperiodic sensitivity of Xanthium leaves of all ages.

Material and Methods

Burs from the species of cocklebur, *Xanthium pennsylvanicum*,[3] were collected from an area around Chicago, Illinois. These burs were soaked overnight and then planted in flats. After the seedlings were about two weeks old they were transplanted into four-inch clay pots or 10-oz. plastic cups as indicated in each experiment. From the time of planting until the time of experimentation the plants were kept in the greenhouse under long days of at least 18 hours of light, supplementing the natural daylight from sunset until 2:00 A.M. The plants were not used for experimentation until they had at least five fully expanded leaves.

After the plants received experimental treatment they were put back on long day for 3 weeks. They were then dissected to determine the presence of flower initiation and the size of the inflorescence. For the degree of magnitude of flowering in Xanthium an

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[3] *Xanthium pen(n)sylvanicum* Wallr., synonym *X. saccharatum* Wallr., as defined in the Eighth Edition of Gray's Manual (1b). Specimens of the plant used in these studies have been filed at the U.C.L.A. Herbarium.

arbitrary scale was used to indicate the stage of flower initiation (11):

0—Vegetative condition.
1—Initiation of the terminal bud, which became dome shaped.
2—Formation of the floret primordia on the periphery of the flower head.
3—Florets initiated on most of the inflorescence.
4—The stage in which the terminal inflorescence was macroscopic.
5—A terminal head 1 mm in diameter.

For each 0.5 mm additional increase in diameter, the arbitrary scale was increased by one unit. To determine the average magnitude of flowering buds, the units of the scale were added and divided by the number of the plants used in the experiment.

Techniques different from those employed by Naylor (10) were used to measure the sensitivity of the leaves and their effectiveness in causing flower initiation. It has been shown that, while the critical dark period is 9.5 hours, the effectiveness of a dark period appears to increase quantitatively as the length of the dark period is increased up to at least 15 hours (11). The effectiveness of the leaf under investigation has been measured in terms of the rate of inflorescence and flower development subsequent to one short day treatment.

In each experiment several lots of 8 or 10 plants were used. On each plant all leaves except one were removed prior to experimental treatment. The position and the area of this leaf were taken as criteria of the age of the leaf. The area of the leaf was calculated by measuring the width and the length of each blade and considering the shape to be triangular. Because of the difficulty of obtaining leaves of exactly the same area, there was a small range of variation in the size among the leaves of a given area designation. The entire plant, including the single attached leaf, was then exposed to one or more short days. In some cases the single attached leaf was removed shortly after treatment, in other cases it was allowed to remain. In each experiment precise details are given of the defoliation techniques and of the length of the dark period and the time allowed for development under subsequent long day conditions. It should be pointed out that the values obtained for rates of development are comparable within an experiment but not between experiments.

In all experiments cited here, the term "flowering" is applied when the terminal bud of the plant has initiated flower primordia or further stages of flowering, i.e., has macroscopic flowers developing flower buds. For convenience of reference in the various experiments, the first expanded leaf is the one nearest to the tip and is the youngest. The leaves are numbered successively down the plant to the oldest leaf near the base. Plants kept continuously under long days were dissected from time to time during the course of the experiments and were always found to be vegetative.

Experimental Results

Sensitivity of the Expanded and Unexpanded Leaves: *Experiment 1:* A preliminary experiment was designed to reexamine in a quantitative manner the effect of age on sensitivity (of the leaf) to the photoperiodic stimulus. Six lots of 8 plants each were selected for treatment. The leaves of the first lot were left intact during the photoinduction period. Four lots were defoliated of all leaves except for a particular pair of alternate leaves. The uppermost pair of fully expanded leaves was retained in lot 2, and successive pairs of expanded leaves were retained in the successive lots. All expanded leaves were cut from the plants of the sixth lot and only very small unexpanded leaves were left on the plant. The leaves were allowed to remain on the plants for the duration of the experiment.

A 20 hour dark period was given to the plants in all 6 lots. Plants so treated were dissected 3 weeks after photoperiodic induction. The results (table I) showed that in lot 2, having only the first pair of expanded leaves, all plants flowered as did all of the non-defoliated plants in lot 1. In lot 3 (characterized by defoliation of all leaves but the third and fourth expanded leaves) only 3 out of 8 plants flowered. The older leaves were relatively ineffective in inducing flowering. An unexpected result was that the sixth lot, which was defoliated except for the very small expanding leaves, gave 4 flowering plants and 4 vegetative (50 % flowering). This latter observation led to the following experiments.

Table I

The Effect of Various Defoliation Treatments on the Response of Xanthium to One Short Day (20 Hours of Darkness). Eight Plants per Treatment

Lot No.	Expanded leaves left on plant	No. of plants flowering	Av. size of * flowering buds
1	All (intact plants)	8	5.6
2	First pair (uppermost)	8	4.2
3	Second pair	3	0.7
4	Third pair	4	1.5
5	Fourth pair	1	0.6
6	None expanded (few expanding) ...	4	1.5

* See text, Col. 1, this page, for the scale used to determine the average magnitude.

Experiment 2: A second experiment was set up to investigate further the sensitivity of the leaves of varying ages. Seven lots of 8 plants each were used. The first lot was defoliated so as to leave on each plant only one young expanding leaf of an area of 10 to 15 cm^2. The second, third, fourth, fifth, and sixth lots retained respectively the first, second, third, fourth, and fifth fully expanded leaf only (fig 1). The range in area of the expanded leaves was from 40 to 45 cm^2. The seventh lot was completely defoliated and used as the control. All 7 lots were given one short day with 15 hours of darkness. When they were

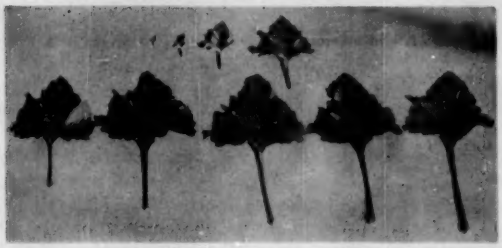

FIG. 1. Leaves at different ages taken from a vegetative Xanthium plant, arranged according to their position on the plant. Upper row, expanding leaves, from left to right: 0.5, 1.8, 9.0, 24.5 cm² in area.

Lower row, expanded leaves, from left to right: first (40 cm² in area), second, third, fourth and fifth (45 cm² in area). The oldest leaf is on the extreme right.

dissected 3 weeks after induction, the results showed that the expanding leaf gave 100 % flowering (fig 2). The first fully expanded leaf gave 87 % flowering, the second 62 %, and the third 12 %. The fourth and fifth fully expanded leaves did not cause a flowering response to the single short day. The completely defoliated control also failed to flower. Thus, the percentage of flowering plants decreased with the advance in leaf age (fig 2).

Experiment 3: Since the youngest leaf investigated in experiment 2 proved to be the most sensitive to the photoperiodic stimulus, a third experiment was designed to determine more precisely the size of the leaf which would give the maximum flowering response. Hamner and Bonner (3) tested the sensitivity of very young leaves, but in all cases the young leaves were removed, presumably soon after they received a photoinductive treatment.

Treatments were therefore included in which the induced leaf was removed from the plant soon after the inductive dark period. The results of Hamner and Bonner could also be interpreted to mean that, while young leaves may be induced, the stimulus may not be translocated until the leaves expand further. Therefore comparisons were also made between plants in which the leaves developing from primordia subsequent to induction were allowed to remain on the plant, and treatments in which all leaves developing after induction were periodically removed. Each lot was defoliated except for one leaf. The leaves remaining on 4 lots of each group consisted of young expanding leaves of different sizes, ranging from the smallest leaves used (range 1 to 2 cm²) to leaves approximately two thirds fully expanded. The youngest fully expanded leaf (range 30 to 45 cm²) was retained on the fifth lot.

Four groups of plants were used, each consisting of 5 lots. Each lot consisted of 10 plants grown in plastic cups. All 4 groups were given a single short day with 20 hours of darkness. The single intact leaf of each plant of one group was removed 2 hours after the end of the long dark period. The leaves of a second group were removed at the same time as those of the first but in addition the leaves subsequently formed were removed before reaching maturity. The treated leaves of a third group were left intact after induction as were those subsequently formed. In the fourth group the treated leaves were left intact and the new leaves which were formed following treatment were removed before reaching maturity.

The plants on which the induced leaf was allowed to remain flowered more markedly than those from which the induced leaf was removed. There was no apparent effect, however, on removal of the leaves which subsequently developed on the plants (table II).

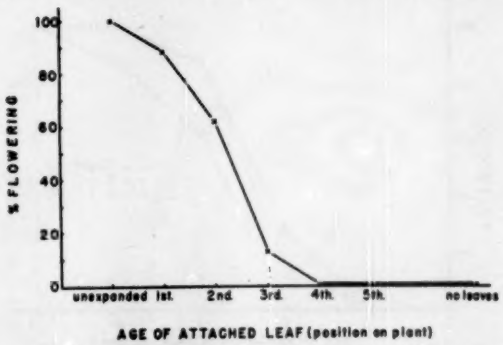

FIG. 2. Effect of age of leaf on percentage flowering. Plants were defoliated to a single leaf and exposed to one short day. The unexpanded leaf had an area of 10 to 15 cm² and the 1st to 5th leaf correspond respectively to those in figure 1.

TABLE II

THE EFFECT OF VARIOUS DEFOLIATION TREATMENTS ON THE RESPONSE OF XANTHIUM TO ONE SHORT DAY (15 HOURS OF DARKNESS). TEN PLANTS PER TREATMENT

Lot No.	Area of treated leaf	Results of treatment: Treated leaf removed* and newly formed leaves: Removed*	Treated leaf removed* and newly formed leaves: Not removed	Treated leaf not removed and newly formed leaves: Removed*	Treated leaf not removed and newly formed leaves: Not removed
	cm²	*percent of plants flowering in each lot*			
1	1–2	0	10	0	0
2	3–5	0	20	70	90
3	8–15	20	20	100	100
4	18–25	20	20	100	100
5	30–45	10	0	50	50
		average stage of development of flowers			
1	1–2	0	0	0	0
2	3–5	0	0.3	3.7	2.8
3	8–15	0.6	0.4	5.6	5.2
4	18–25	0.4	0.6	4.6	5.0
5	30–45	0.2	0	1.8	3.3

* Treated leaves removed 2 hours after end of long dark period. Newly forming leaves removed before they reached maturity.

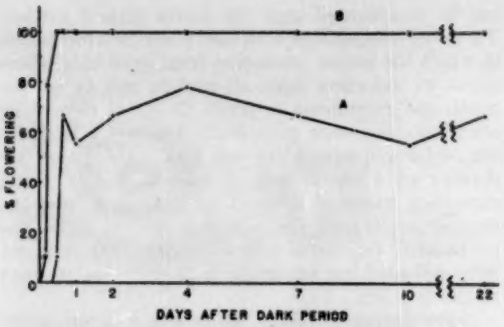

FIG. 3. Effect of time of removal of induced leaf on flowering response. All plants were defoliated except for a single leaf, exposed to one short day, and treated leaves removed at times indicated. Curve A represents responses in which the treated leaf was 3 to 5 cm² and for curve B the treated leaf was 7 to 15 cm².

The smallest leaf did not respond to a single short day. However the larger leaves all gave a high flowering response. The maximum flowering was with leaves one half to two thirds fully expanded. The first (youngest) fully expanded leaves gave a response less than those not fully expanded. Even when the leaves were removed 2 hours after the 20 hour dark period, the expanding leaves of intermediate size gave 20 % flowering. This finding led to a more precise investigation of transport of the flower promoting stimulus from the leaf.

Experiment 4: The next experiment was designed to determine the length of time required for the flowering stimulus to move out of the leaf. One hundred ninety-eight plants were divided into 2 groups. Plants in one group were defoliated to a single unexpanded leaf having a range in blade area of 3 to 5 cm², and those of the second group to a single unexpanded leaf of 7 to 15 cm². All plants were given one short day with 15 hours of darkness, then returned to long days. Each group was divided into 11 lots of 9 plants each. The attached leaf was defoliated in respective lots 0, 4, 8, 12, 16, or 24 hours, or 2, 4, 7, or 10 days, after the end of the long dark period. The last lot retained the leaf until the day of dissection, viz., 22 days after induction.

The results of this experiment are given in figure 3. The group of plants in which the treated leaf was the smaller (Curve A) exhibited a maximum response of 60 to 80 % flowering. This maximum response appeared to occur if the treated leaf was retained for 16 hours or more after the end of the long dark period. The group of plants in which the treated leaf was the larger (Curve B) gave 100 % flowering if the treated leaf was retained for 12 hours or longer following the long dark period. The curves for magnitude of flowering are not presented because they coincide with those for the percentage of flowering. Leaf area measurements showed a very slight increase within 12 to 16 hours after induction. Flowering in the plants with the smaller leaf never reached 100 % during the period of 3 weeks in which the experimental leaf became fully expanded even though other new leaves were formed and went through their development during this time.

This experiment indicates that the leaf age is critical for the formation of the stimulus only during the photoinductive period. It also demonstrates that the stimulus may move out of the leaves with great rapidity after the inductive dark period.

SENSITIVITY TO PHOTOPERIODIC INDUCTION OF BUDS OF DIFFERENT AGES: The response of small unexpanded leaves to short days was studied in experiment 5 using buds in different stages of development. The plants which were used in this experiment were about 6 weeks old and were grown in plastic cups. The entire top of the plants above the cotyledonary node was cut off, the cotyledons were removed and only one dormant cotyledonary bud was left on each plant. The bud began to grow immediately after this treatment and in this discussion its age is expressed as the number of days after trimming. The plants were divided into 6 groups of 24 plants each and 1, 4, 7, 10, 13, or 16 days later, plants of the respective groups were exposed to photoinductive treatment. Thus, the day after the plants had been trimmed down to one dormant bud, the first group of 24 plants was exposed to 16 hours of darkness. The next day the plants of this group were divided into 3 lots. One lot received only this one short day treatment. The second lot was given 2, and the third lot received 3 short days. All plants were then returned to long day conditions. Each successive group was treated in the same way after 4, 7, 10, 13, or 16 days had elapsed after the trimming. The plants of each lot were dissected 40 days after the short day treatment.

The results, presented in figure 4, show that the buds responded to one short day when they were 10 days old. When they were 16 days old they gave 100 % flowering. With more than one short day they responded faster. The relationship between the age of the bud, the number of short days of treatment,

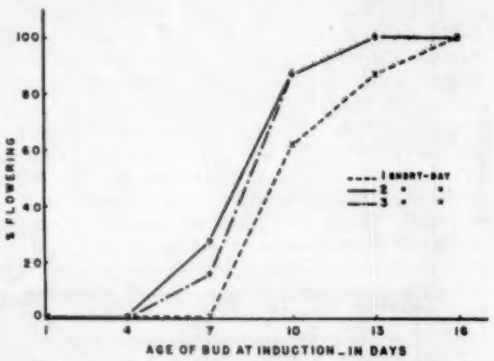

FIG. 4. Effect of photoperiodic induction on buds of different ages. Each short day consisted of 16 hours of darkness and 8 hours of daylight. Eight plants were used per treatment.

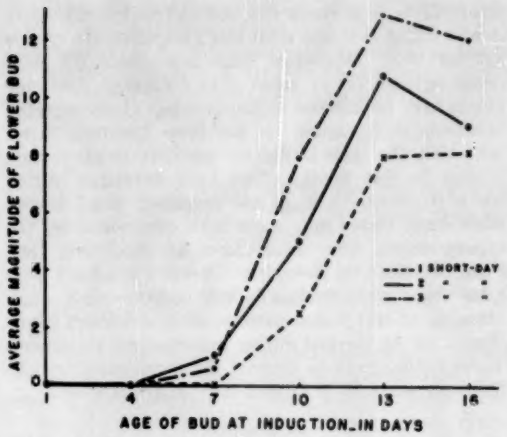

FIG. 5. Effect of photoperiodic induction on buds of different ages. Each short day consisted of 16 hours of darkness and 8 hours of daylight. Eight plants were used per treatment.

and the magnitude of the response are shown in figure 5. Maximum flowering was observed when the plants were treated for 3 days starting on the thirteenth day. There was no flowering when the buds were 1 or 4 days old when treated, but when they were 7 days old they showed 16 % or 28 % flowering when given 2 or 3 short days respectively.

In order to compare the flowering response to the stage of morphological development the length of the bud, number of visible leaves, the area of the visible leaves, and the number of leaf primordia were measured during the course of the bud development. At the age of 10 days when the percentage of flowering response was higher than 60 % (fig 4) the buds were 9 mm long (fig 6) with 3 to 4 visible leaves of an average total area of 0.22 cm² and 4 leaf primordia (fig 7). At the age of 13 days the flowering was 100 % and the buds were 14.3 mm long (fig 6), and there were 4 visible leaves of 1.12 cm² average total area (fig 7) and 4 leaf primordia. On the sixteenth day the branch was 27 mm long and there were 4 to 5 visible leaves of 2.26 cm² average total area, and 4 leaf primordia.

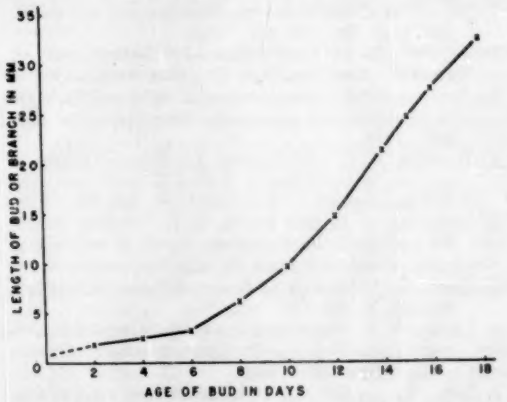

FIG. 6. Length of buds in experiment 5 (fig 4 and 5). Readings are average of 20 buds.

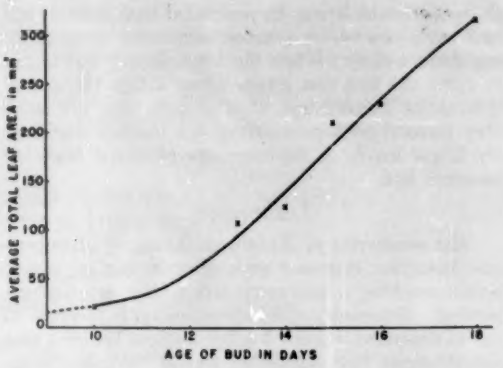

FIG. 7. Average total leaf area of buds in experiment 5 (fig 4 and 5). Each measurement was of buds on 5 plants.

The results indicate that a total area of 0.2 cm² is sufficient to cause response to 16 hours of darkness in a single photoinductive cycle. This is a very small amount of leaf tissue to respond to photoperiodic induction, and it seemed surprising when compared with previous work with young unexpanded leaves which had to have at least 3 to 5 cm² in order to respond.

The question arose whether the physiological age of the leaf was more significant than the area of the leaf in determining sensitivity to photoperiodic stimuli. An experiment was designed to determine the rate of growth of a leaf in a terminal bud. From Xanthium seedlings grown in pots, 5 plants were chosen having a leaf of the area 0.1 cm². Each leaf was measured daily for 12 days. Figure 8 shows that by the twelfth day the leaf reached full size. From

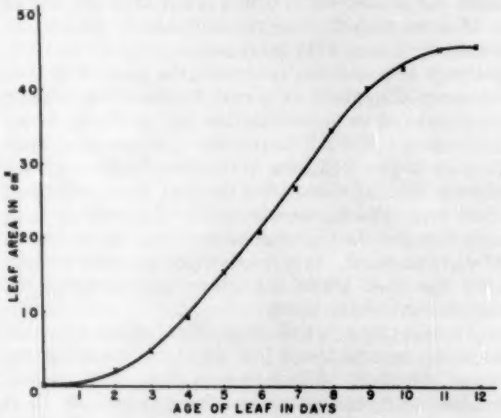

FIG. 8. Growth of untreated Xanthium leaf. A leaf of 0.1 cm² was chosen on each of 5 vegetative plants. Leaf blade area was measured daily until fully expanded.

this experiment it can be concluded that a small leaf (0.1 cm^2) reaches its greatest sensitivity after growing about 5 days. When the cotyledonary bud begins to grow the first few leaves never attain the size of subsequent leaves; but, even though they are small they respond photoperiodically in a manner similar to the larger leaves of the same age produced from the terminal bud.

Discussion

The sensitivity of Xanthium leaves to photoperiodic induction increases with aging of the expanding leaves reaching a maximum when they are half expanded. Sensitivity then decreases with increase of age of expanded leaves. Earlier workers believed that the youngest fully expanded leaf is the most sensitive to photoperiodic induction. Results reported here indicate that the half-expanded leaf is the most sensitive.

It is of importance to know which is the most sensitive leaf to the photoperiodic induction in order to study the photoperiodic changes in relation to biochemical changes. In order to follow the changes in leaves of a plant which has been induced it is important to choose a leaf which is photoperiodically sensitive. In preliminary experiments on auxin and growth inhibitor reported by Khudairi and Bonde (5) only the expanded leaves of Xanthium were taken for analyses. Results were inconsistent and the cause was not realized until the discovery of the relation between age of the leaf and its sensitivity to photoperiodic induction. Subsequently the leaves were chosen very carefully according to their age.

These experiments also make possible certain conclusions about the translocation of the stimulus from leaves to buds. It has been believed (4) that it takes about 4 days for the floral stimulus to move from the leaves through the stem to cause flowering in Xanthium. In these experiments it is shown by removing the induced leaves at different time intervals after the dark period of a short day that the stimulus begins to move out of the leaf in 2 to 4 hours after the end of a 15 hour period. This corresponds to results obtained by Raven (11) using intact plants. Two hypotheses may be made to explain the interval of time necessary after the dark period, for the stimulus to be produced and to move from the leaf in effective concentrations. First, it is possible that chemical reactions leading to formation of the stimulus have to take place in the leaf tissue after the long dark period and these may take appreciable time. Secondly, it may take time for the stimulus to move out of the leaf in effective amounts. It is possible that exposure to light after the dark period accelerates movement of the stimulus out of the leaves.

Dormant buds, which do not develop into branches normally, may be forced into growth by removing the top of the plant. The leaves on these buds become sensitive to short days after seven days growth. It is apparently not the leaf size which determines its sensitivity, but the physiological age of the leaf. Thus, the normally developing leaf in a terminal bud takes about 12 days to reach full size (45 cm^2 area), while it was found that the total leaf area of leaves of this age on newly developed buds was about 0.5 cm^2. Some workers (7, 8) claim that defoliated short-day plants may be induced to flower when given repeated favorable day lengths. It has been assumed, therefore, that the stem is slightly sensitive to short day. It may be that small axillary buds developed during the experimental period and produced small leaves. Such small leaves may have been overlooked by the experimenters, who would have assumed that they have no effect on flowering. Results obtained here with the newly developed buds indicate that small amounts of leaf tissue present on a defoliated plant should not be ignored during experimental treatment. These results seem to support the hypothesis that the flowering stimulus is formed only in green leaves.

Summary

1. Single leaves of different ages were left on Xanthium plants and given one short day of either 15 or 20 hours darkness. The age was determined by the position of the leaf on the plant and by the leaf area.

2. The youngest leaf blades (ranging in area 1 to 2 cm^2) on the plant gave no flowering response, but the response increased with the age of the expanding leaf, reaching a maximum in the leaves of an area approximately one half that of a fully expanded one. A reversal occurs at this point and the sensitivity decreases in the successively older ones.

3. When the induced single leaves were defoliated at different intervals after the end of the inductive period, it was found that with the most sensitive leaves, flowering could be detected when the leaves were removed within 4 hours after 15 hours of darkness, and 2 hours after a 20 hour dark period.

4. Plants with one dormant bud may be induced to flower when the buds are 7 days old and give 100 % flowering after 13 days.

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CARBOHYDRATE METABOLISM AND OXALIC ACID SYNTHESIS BY BOTRYTIS CINEREA[1,2]

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Most of the work on the metabolism of the fungi has dealt with the production of organic acids and other compounds formed via pathways which are, presumably, shunts from the main respiratory cycles (6, 14). Few attempts have been made to integrate these pathways into over-all metabolic systems. This study will follow the formation of oxalic acid, which appears to be a metabolic end product, from glucose through the respiratory system of *Botrytis cinerea* Pers.

Materials and Methods

In all experiments a strain of *Botrytis cinerea* (7) was grown at room temperature in 250-ml Erlenmeyer flasks containing 100 ml of the following medium: $NaNO_3$, 2.0 gm; KH_2PO_4, 1.5 gm; $MgSO_4 \cdot 7H_2O$, 1.0 gm; Hoagland's A–Z trace element solution (9), 1.0 ml; glucose, 20.0 gm; H_2O to make 1 liter. Inoculum for flasks was provided by growing the fungus in Petri dishes on the above medium solidified with 2% agar. Uniform discs were cut out with a cork borer 7 mm in diameter and each flask inoculated with 1 disc. Unless otherwise noted, the cultures were mechanically shaken during incubation (10 days). The pellets of mycelium which formed were then washed and shaken for 3 days in numerous changes of the above liquid medium without a carbon source. These pellets of starved mycelium were used in all respiratory and biosynthesis experiments (tables II to IV).

Oxalic acid was removed from culture filtrates essentially according to Pucher, Vickery, and Wakeman (12). The filtrates were acidified with H_2SO_4 and the oxalic acid precipitated with saturated $Ca(NO_3)_2$. One ml of the oxalic acid solution obtained was placed in a Warburg vessel and, at zero time, 0.5 ml of acid permanganate solution (8% $KMnO_4$ in 7 N H_2SO_4) was tipped in. Under these conditions 2 moles of CO_2 were formed from each mole of oxalic acid. In numerous tests with known concentrations of oxalic acid, it was found possible to obtain an accuracy of about 5% with this method. Of all the organic acids discussed in this paper, only citric interfered in this method. However, no trace of citric acid was found in any filtrates of this organism when they were analyzed chromatographically. Glucose was determined by the procedure of Folin and Malmros (5), nitrate by a phenoldisulfonic acid method (13), and organic acids, qualitatively, by the method of Lugg and Overell (8). All manometric studies were conducted at 26°C, pH 4.0, using standard Warburg techniques. The vessels contained 3.0 ml of the medium previously described, with added substrate, where indicated, and KOH with filter paper wicks were used in the center well.

Results

A 6-week time-course study was undertaken to investigate the pattern of oxalic acid formation and the physiological changes accompanying its synthesis. Each value in figure 1 represents the results of duplicate analyses for oxalic acid of combined filtrates and in addition the average mycelial weights of 6 standing cultures. The pH of the cultures rose slowly from 4.2 at the time of inoculation to 6.5 at the end of 6 weeks. Oxalic acid formation does not appear to accompany the growth of the fungus, since most of the oxalic acid was produced before the log phase of growth. There was only a slight increase in total oxalic acid during the second week, and it began to fall off slightly after this time. Oxalic acid synthesis apears to be concomitant with the rapid utilization of glucose. At the end of the 6 week period, the growth curves began to level off although only about 50% of the carbohydrate and 30% of the nitrate available in the medium had been utilized. Cessation of growth may have been due to production of "stale products" rather than to any limitation of available nutrient.

Figure 2 shows the rates of O_2 uptake of mats of mycelium of various ages, each value obtained from a random sample of segments of mats taken from several standing cultures. The segments were cut into small squares and shaken in Warburg vessels, each vessel containing about 20 mg dry weight of mycelium suspended in 3.0 ml of medium containing glucose (5×10^{-3} M). The Q_{O_2} reached a peak at 2 weeks and subsequently fell off rapidly, leveling off by

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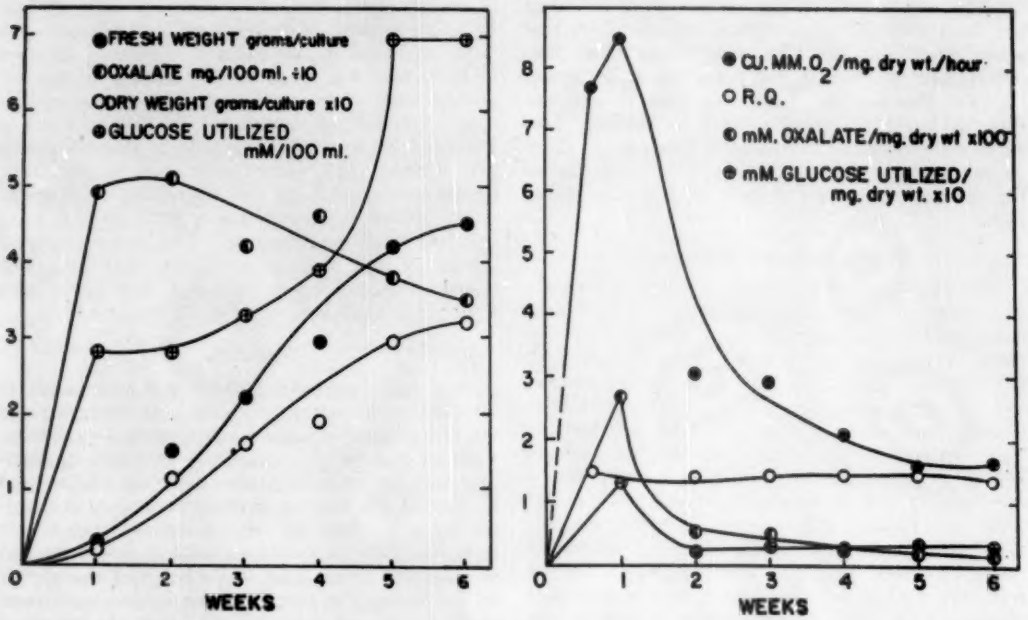
5 weeks. The respiratory quotient (R.Q.) remained above unity throughout the 6-week period. The curves for glucose utilization and oxalic acid formation per unit dry weight parallel each other, indicating a relationship between the amount of glucose utilized and the amount of oxalic acid formed. The amount of oxalic acid produced by this organism was independent of the pH of the medium (table I).

To investigate the pathways of carbohydrate oxidation by *Botrytis*, hexoses, pentoses, trioses, and selected phosphorylated intermediates were used as respiratory substrates (table II). The figures presented here and in table III were calculated from the mean rates of O_2 uptake obtained from at least 2 separate experiments. Both endogenous and substrate respiration were measured in duplicate vessels in each experiment, each vessel containing 8 to 12 mg dry wt. of mycelium. Under the conditions of growth used here the Q_{O_2} of starved pellets of mycelium originating from different cultures varied from about 2.0 to 4.0. However, in any one experiment the tissue used was all obtained from the same culture and the variation about the mean Q_{O_2} was $\pm$ 0.5. Under these conditions, the percentages of stimulation of O_2 uptake obtained in duplicate experiments were in close agreement (10 %). In every case where stimulation of O_2 uptake is reported, the lower substrate Q_{O_2}, was significantly higher than the upper endogenous Q_{O_2}.

Stimulation of O_2 uptake with glucose, gluconic acid, phosphogluconic acid, and ribose-5-phosphate suggests that glucose oxidation proceeds via direct oxidation through phosphorylated intermediates to pyruvic acid. Increased O_2 uptake was obtained with xylose and arabinose, but ribose was not oxidized. All tested phosphorylated hexose sugars as well as 5-keto-gluconic acid failed to increase the rate of respiration. Increased O_2 uptake was obtained with 3-phosphoglyceraldehyde but not with α- or β-glycerophosphate. In all instances where addition of substrate failed to enhance O_2 uptake, the possibility that the substrate failed to enter the cells cannot be eliminated. An acetone powder extract of mycelium prepared according to Arnon (1) contained glyceraldehyde phosphate dehydrogenase, which was completely DPN dependent, but did not appear to contain aldolase. Over 80 % inhibition of both endogenous and glucose respiration was obtained with sodium fluoride (10^{-3} M, pH 4.0). These results indicate that the oxidation of triose to pyruvic acid proceeds via the Meyerhof-Embden pathway. Fermentation was lacking, since neither CO_2 nor lactic acid was produced anaerobically. However, lactic acid was found in some aerobic cultures after several weeks of growth.



FIGS. 1 and 2. Fig. 1 (*left*). Growth, glucose utilized, and oxalic acid produced by *Botrytis cinerea* over a 6-week period. Fig. 2 (*right*). Oxygen uptake, respiratory quotient, oxalic acid formed, and glucose utilized per unit dry weight of *Botrytis cinerea* over a 6-week period.

Paper chromatography of the filtrates from the cultures showed the presence of pyruvic, succinic, fumaric, and malic acids—all intermediates of the Krebs cycle. In addition, glycolic and lactic acids were found. Various Krebs cycle acids, pyruvic, citric, α-ketoglutaric, and succinic (table III), stimulated O_2 uptake of starved pellets of mycelium.

TABLE I

OXALIC ACID FORMATION BY BOTRYTIS CINEREA GROWN FOR 4 WEEKS AT VARIOUS HYDROGEN ION CONCENTRATIONS

INITIAL pH	FINAL pH	OXALIC ACID (MG/100 ML MEDIUM)
2.0	3.0	54
2.6	3.6	38
3.9	3.8	58
4.8	3.2	68
6.7	5.8	50

Malic acid did not significantly increase O_2 uptake. Malonic acid (10^{-2} M, pH 4.0) inhibited O_2 uptake 37 %, this inhibition being completely relieved by succinic acid (5×10^{-2} M). Stimulation of the respiration of starved mycelium with most Krebs cycle intermediates, as well as the inhibition of succinic dehydrogenase with malonic acid and reversal of this inhibition with succinic acid, suggest that a Krebs cycle is functioning in this microorganism. There was no significant difference in O_2 uptake with oxalic acid as compared with the endogenous respiration. Since long-term growth experiments also demonstrated that oxalic acid could not be utilized as a carbon source, it appears that oxalic acid is an end product of the metabolism of *Botrytis cinerea*.

In order to obtain information on the terminal oxidase system of this organism, experiments on the effect of sodium azide on oxygen uptake were performed. Sodium azide (10^{-3} M, pH 4.0) showed 85 % inhibition of both glucose and endogenous respiration, indicating that the terminal oxidase system is probably mediated by heavy-metal enzymes.

Experiments to determine the pathway of oxalic acid synthesis were conducted. Approximately equal weights of pellets of starved mycelium were incubated at 30°C for 3 hours in 10 ml of standard medium with various organic acids. As a control the organism was incubated in this medium without substrate. All experiments were done in duplicate. At the end of the incubation period, trichloroacetic acid was added to each flask to a final concentration of 15 %. The flasks were allowed to stand for 30 minutes; the media were filtered, and then analyzed for oxalic acid. High yields of oxalic acid were obtained with malic acid as the substrate. Considerable oxalic acid was also formed from oxaloacetic acid. No significant amounts of oxalic acid were produced when acetic, glyoxylic, glycolic, or formic acids were used as precursors.

TABLE II

OXYGEN UPTAKE OF BOTRYTIS CINEREA WITH VARIOUS SUGARS AND SUGAR DERIVATIVES AS SUBSTRATES *

SUBSTRATE	O_2 UPTAKE AS PERCENT OF ENDOGENOUS
Control	100
Glucose	170
Fructose	160
Glucose-1-phosphate	97
Glucose-6-phosphate	103
Fructose-1,6-diphosphate	104
Gluconic acid	210
5-Ketogluconic acid	95
Phosphogluconic acid	190
Ribose **	107
Xylose **	190
Arabinose **	138
Ribose-5-phosphate **	160
3-Phosphoglyceraldehyde **	140
α-Glycerophosphate **	87
β-Glycerophosphate **	90

* 5×10^{-3} M concentration; pH 4.0.
** 1×10^{-3} M concentration.

TABLE III

OXYGEN UPTAKE OF BOTRYTIS CINEREA WITH VARIOUS ORGANIC ACIDS AS SUBSTRATES *

SUBSTRATE	O_2 UPTAKE AS PERCENT OF ENDOGENOUS
Control	100
Pyruvic	224
Acetic	134
Citric	272
α-Ketoglutaric	156
Succinic	195
Malic	110
Glycolic	121
Oxalic	96

* 5×10^{-3} M concentration; pH 4.0.

Since malic and oxalacetic acids appeared to be precursors of oxalic acid, experiments were conducted using these acids as well as oxalosuccinic and succinic acids. These experiments were conducted essentially in the manner described above. Controls for these experiments consisted of flasks without mycelium and flasks containing boiled mycelium. The boiled mycelium was used to estimate the oxalic acid in the tissues at zero time. At the end of a 2 hour incubation period, the dry weights of the mycelia were obtained and the filtrates analyzed for oxalic acid (table IV). Here, as in preliminary experiments, the highest yields of oxalic acid were obtained from malic acid. Considerable yields of oxalic acid were obtained from oxaloacetic and oxalosuccinic acids and relatively small yields from succinic acid.

In experiments with oxaloacetic and oxalosuccinic acids, the spontaneous decarboxylation of these com-

TABLE IV

OXALIC ACID FORMATION BY BOTRYTIS CINEREA IN 2 HOURS ON VARIOUS SUBSTRATES, pH 3.0

SUBSTRATE	μM SUBSTRATE PER 25 ML MEDIUM	μM OXALIC ACID FORMED PER 25 ML MEDIUM	μM OXALIC ACID FORMED PER MG DRY WEIGHT	MOLAR PERCENTAGE CONVERSION
Malic	125	16.6	0.17	13.3
Oxalosuccinic	25	2.2	0.03	8.8
Oxaloacetic	125	9.0	0.11	7.2
Succinic	125	4.0	0.03	3.2

pounds in aqueous solution to pyruvic and α-ketoglutaric acids, respectively (11) must be taken into account. Neither of these breakdown products proved to be precursors of oxalic acid. Thus the molar percentage conversion figures presented in table IV are probably low, if we consider the lowering concentrations of oxaloacetic and oxalosuccinic acids available for enzymatic action as the experiment progressed.

Discussion

The findings presented here suggest that glucose is being oxidized directly to gluconic acid which is phosphorylated (2) to phosphogluconic acid and then proceeds via phosphorylated intermediates to a triose. The phosphorylated pentose in this pathway could be derived from xylose or arabinose. Although ribose was not oxidized, ribose-5-phosphate may be the phosphorylated pentose formed by pathways other than direct phosphorylation (2, 3). Increased O_2 uptake with phosphoglyceraldehyde, the presence of glyceraldehyde phosphate dehydrogenase, and the inhibition of respiration with fluoride suggest that the pathway from triose to pyruvate proceeds according to the Meyerhof scheme. The failure of phosphorylated hexoses, 5-ketogluconate, and phosphoglycerate to enhance O_2 uptake cannot be interpreted since it is probable that these compounds failed to enter the cells.

The stimulation of respiration with acids of the Krebs cycle, the detection of Krebs cycle acids in culture filtrates, plus the inhibition of respiration with malonic acid and the reversal of this inhibition with succinic acid indicate that a Krebs cycle is operating. Enhanced O_2 uptake with acetic and glycolic acids suggests an additional pathway, possibly from succinic to acetic, then to glycolic, glyoxylic, and formic acids (10). Evidence presented here suggests that the main route of oxalic acid formation proceeds via direct oxidation of malic acid. Nord and Vitucci (10) reached this conclusion on the basis of long-term growth experiments. This pathway has now been demonstrated in short-term biosynthesis studies. Failure to obtain appreciable oxalic acid synthesis from glycolic, glyoxylic, and formic acids eliminates the possibility that the pathway from malic to oxalic acid via these intermediates (10) is operating to any apreciable extent in this organism. Some oxalic acid also can be formed by dismutation of oxalosuccinic and oxaloacetic acids. The oxalic acid obtained from succinic acid was probably formed via oxidation of the succinic to malic acid.

A long-term study of oxalic acid formation has shown that most of the acid is formed during the first week of growth. During this period malic acid could not be found in the culture filtrates, although it was the acid most readily found in older cultures. Oxalic acid formation does not take place during the period of maximum growth of the organism, but only during the period when the organism is highly oxidative. In view of the low rate of oxidation of malic acid even in vigorous cultures and the failure of malic acid to accumulate during the early period of growth, it would seem that the malic-to-oxalic route is operating. The formation of oxalic acid then serves as a mechanism for the removal of excess malic acid from the system and permits the Krebs cycle to operate at a rapid rate. The formation of oxalic acid by this organism virtually ceases after 1 week in culture, possibly owing to inactivation of the enzyme system involved or to the accumulation of the end product of the reaction. The pathway from malic to oxalic acid appears to be irreversible since oxalic acid could not be used for growth or as a respiratory substrate. The termination of oxalic acid formation coincides with a sharp decline in O_2 uptake. Presumably all the malic acid being oxidized after this time is going through the Krebs cycle.

The pH value of the medium does not appear to affect oxalic acid formation. It has been reported that alkalinity of the medium is the chief factor in the accumulation of oxalic acid (4). This situation may apply to organisms which utilize the free acid and not the salts, in which cases the alkali serves to trap the acid and make it unavailable to the organism.

Summary

Experimental evidence suggests that glucose is oxidized directly to gluconic acid, and then, via phosphogluconate, pentose phosphate, and triose phosphate, to pyruvate. The organism does not produce CO_2 or lactic acid anaerobically. A Krebs cycle appears to be functioning. Inhibition of respiration with azide suggests that the terminal oxidase system may be mediated entirely by heavy-metal enzymes.

Botrytis cinerea is capable of synthesizing oxalic acid, most of which is formed during the first week of growth. Oxalic acid synthesis does not parallel growth of the fungus but occurs when the organism is in a phase of high oxidation. A decline in O_2 uptake and in glucose utilization is coincident with the cessation of oxalic acid formation. The main pathway of oxalic acid synthesis is via direct oxidation of malic acid, although some oxalic acid may be formed by the hydrolytic dismutation of oxaloacetic and oxalosuccinic acids. Oxalic acid cannot be utilized as a respiratory substrate or as a carbon source for growth.

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NATURE AND DISTRIBUTION OF GLYCOLIC ACID OXIDASE IN PLANTS[1,2]

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Clagett et al (3, 4) reported the presence of a soluble enzyme in the green parts of higher plants which rapidly oxidizes glycolic and L-lactic acids, and Kolesnikov (6) also observed a rapid oxidation of glycolic acid added to ground barley leaves. Glycolic acid was oxidized more rapidly than L-lactic acid, and, among many other compounds tested, only DL-α-hydroxy-n-butyric acid was oxidized and this at a very slow rate (3). Tolbert et al (12) further purified the enzyme from tobacco leaves and identified the product of L-lactic acid oxidation as pyruvic acid. With both crude and purified enzyme preparations, the first product of glycolic acid oxidation was glyoxylic acid, which also was oxidized slowly. Oxidation of glycolic acid by purified preparations yielded formic acid and carbon dioxide, but the end products formed by crude preparations were not identified. Tolbert and Burris (11) later reported that the enzyme was absent from etiolated plants but was activated by light; the activation did not follow chlorophyll formation.

Zelitch and Ochoa (19, 20) have obtained a catalase-free enzyme preparation from spinach leaves which was 74 to 80% pure by electrophoretic and ultracentrifugal criteria and which oxidized both glycolic and L-lactic acids. Apparently only one enzyme was involved in the oxidation of the two acids. They showed that riboflavin monophosphate is the prosthetic group, and that hydrogen peroxide is produced in the oxidation of glycolic acid to glyoxylic acid and in the oxidation of L-lactic acid to pyruvic acid. In the absence of catalase, glyoxylic and pyruvic acids are oxidized non-enzymatically by hydrogen peroxide to formic acid plus carbon dioxide and to acetic acid plus carbon dioxide, respectively. Kenten and Mann (5) also had demonstrated the production of hydrogen peroxide from the enzymatic oxidation of lactic and glycolic acids, and had shown that glyoxylic acid is enzymatically oxidized to oxalic acid in the presence of catalase.

Glycolic and L-lactic acids are present in a wide variety of plants. Reports of their occurrence have been summarized by Rabinowitch (8) and Thimann and Bonner (10). Moreover, Benson and Calvin (2) have found that glycolic acid is one of the early products of photosynthesis. Tolbert and Cohan (13) demonstrated the incorporation of considerable C^{14} into glycine, polyglycine, and serine from C^{14}-labeled glycolic acid added to crude barley juice. Schou et al (9) supplied C^{14}-labeled glycolic acid to Scenedesmus and observed particularly high aerobic incorporation of the C^{14} into phosphoglyceric acid, serine, glutamic acid, and hexosephosphates in the dark, and into phosphoglyceric acid, sucrose, serine, glutamic acid, and malic acid in the light. Anaerobically in the dark, much C^{14} accumulated in glycine.

This paper reports the occurrence of glycolic acid

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oxidase[4] in a variety of plants and describes some of the characteristics of the enzyme.

Methods and Materials

Measurements of O_2 consumption were made in Warburg constant volume respirometers at 30°C (16). The 2.5 ml of solution in the main compartment of the flask included 1 ml of 0.1 M phosphate buffer or 0.1 M tris-hydroxymethylaminomethane buffer, enzyme preparation, any material being tested, and water to volume. One half ml of 0.02 M glycolate or 0.1 M DL-lactate (or water for controls without added substrate) was added from the sidearm. The center well contained 0.15 ml of 20 % KOH to absorb CO_2. For rate measurements the initial O_2 consumption was determined.

Activity and purity of enzyme preparations are expressed as $Q_{O_2}(N)$ values; that is, as the μl O_2 taken up per hour per mg tissue nitrogen in the enzyme preparation. Total nitrogen was determined by the Umbreit and Bond (15) modification of the Kjeldahl method, with copper selenite as the digestion catalyst.

The activity of the enzyme could also be measured in Thunberg tubes by determining photometrically the rate of the anaerobic reduction of 2,6-dichlorophenolindophenol (16).

For the survey of plants for glycolic acid oxidase activity and for the study of the enzyme in barley, the leaves of the plants were ground in a mortar with quartz sand, or with a Nixtamal mill. The brei was immediately chilled in an ice bath; it was strained through a double layer of cheesecloth, adjusted to pH 8, and centrifuged at 2500 × g for 10 minutes. The supernatant fluid was used as a crude enzyme preparation and is referred to here as "plant juice."

Because of the high stability and abundance of the enzyme they contain, tobacco leaves were used as the source of enzyme for purification by salt fractionation. Tobacco juice was fractionated by precipitation with Na_2SO_4 and $(NH_4)_2SO_4$ according to Tolbert et al (12). This enzyme preparation, which was purified about tenfold to give a $Q_{O_2}(N)$ of around 2000, is referred to as the "salt-precipitated enzyme." It can be stored at from 2 to 5°C for several weeks without appreciable loss of activity. Upon dialysis, the $Q_{O_2}(N)$ increases to about 2500, but the enzyme loses stability so that its activity decreases by about 50 % upon storage at from 2 to 5°C for 96 hours.

The third type of enzyme preparation (referred to as the "purified spinach enzyme") was the purified enzyme from spinach leaves, prepared by Zelitch and Ochoa (19). Riboflavin monophosphate was added to reconstitute the enzyme.

For the study of light activation, etiolated barley seedlings were grown inside a light-proof box in a dark room. A panchromatic photographic film was placed on the flat containing the barley seedlings to detect any light that might fall on the barley seedlings; if the film was exposed the barley seedlings were discarded. The barley juice from etiolated plants was prepared in the darkroom, and the Warburg flasks were filled, placed on the manometers, and covered with tinfoil before being brought into the light.

Glycolic acid was obtained from the Matheson Co., DL-lactic acid from Fisher Scientific Company, and iodoacetic acid and phenylmercuric nitrate from Eastman Kodak Company. *p*-Chloromercuribenzoic acid was prepared by the alkaline permanganate oxidation of tolylmercuric chloride (18). Iodoacetamide was synthesized from chloroacetamide by a modification of the method of Von Braun (17).

Experimental Results

Survey of Plants for Glycolic Acid Oxidase: To survey the distribution of glycolic acid oxidase in plants, the crude juices from the leaves of a wide variety of plants were tested for their ability to support uptake of O_2 with added glycolic or lactic acids. Phosphate buffer was used, and the pH was adjusted to 8.3 for glycolate and 7.6 for lactate. All leaves tested (except for Japanese yew, okra, and box elder, which gave little juice and therefore unsatisfactory preparations) yielded active glycolic acid oxidase. Rhubarb juice was much more acidic (pH 3.9) than the other plant juices tested, and unless the leaves were vacuum infiltrated with pH 8.9 phosphate buffer before they were ground, this high acidity quickly inactivated the enzyme. In general, activity with glycolic acid as a substrate was higher than that with lactic acid. However, some preparations with a low concentration of enzyme were more active with lactic acid. The data are summarized in table I.

Inhibition of Glycolic Acid Oxidase by Sulfhydryl Inhibitors: The presence of free sulfhydryl groups in the glycolic acid oxidase molecule was indicated by manometric demonstration that the initial rate of O_2 uptake was inhibited by iodoacetate, iodoacetamide, phenylmercuric nitrate, and *p*-chloromercuribenzoate (1, 7). In all tests, these inhibitors were added to a buffered enzyme preparation (either the barley juice or the purified spinach enzyme previously reconstituted by the addition of riboflavin monophosphate) and incubated for 30 minutes at 30°C before addition of substrate. All inhibition studies, except those with purified spinach enzyme, were made with

[4] Zelitch and Ochoa (1953) have called this enzyme glycolic acid oxidase, and Kenten and Mann (1952) have applied the term alpha-hydroxy acid oxidase. To avoid a multiplicity of names for a single enzyme we have adopted the suggestion of a reviewer of this paper to apply the term glycolic acid oxidase. It should be noted, however, that the enzyme is not an oxidase in the classical sense of a metalloprotein enzyme which activates oxygen and forms water, for the enzyme is actually a flavoprotein which forms hydrogen peroxide. It has the properties commonly attributed to a dehydrogenase, i.e., it activates hydrogen and reduces dyes such as 2,6-dichlorophenolindophenol in the absence of oxygen. The lack of a uniform terminology for enzymes leads to the confusion evident here, for it is customarily acceptable to refer to an enzyme as xanthine oxidase or xanthine dehydrogenase, and to the enzyme in question as glycolic acid oxidase or glycolic acid dehydrogenase.

TABLE I

UPTAKE OF OXYGEN BY CRUDE JUICES FROM THE LEAVES OF VARIOUS PLANTS WITH GLYCOLIC ACID OR LACTIC ACID FURNISHED AS SUBSTRATE

PLANT			$Q_{O_2}(N)$		RATIO OF ACTIVITIES GLYC./LACT.
COMMON NAME	SCIENTIFIC NAME	FAMILY NAME	GLYCOLATE	LACTATE	
Broccoli	*Brassica oleracea*, var. *botrytis*	Cruciferae	105	95	1.10
Cabbage	*Brassica oleracea*, var. *capitata*	Cruciferae	111	96	1.16
Kohlrabi	*Brassica caulorapa*	Cruciferae	70	62	1.13
Asparagus (mature)	*Asparagus officinalis*, var. *altilis*	Liliaceae	377	316	1.19
Swiss chard	*Beta vulgaris*, var. *cicla*	Chenopodiaceae	236	135	1.73
Squash	*Cucurbita maxima*	Cucurbitaceae	70	54	1.30
Lima bean	*Phaseolus limensis*	Leguminosae	90	72	1.25
Carrot	*Daucus carota*	Umbelliferae	55	60	0.92
Rhubarb	*Rheum hybridum*	Polygonaceae	50	70	0.71
Raspberry	*Rubus idaeus*	Rosaceae	68	68	1.00
Lily	*Lilium tigrinum*	Liliaceae	244	160	1.52
Iris	*Iris germanica*	Iridaceae	231	164	1.41
Zinnia	*Zinnia elegans*	Compositae	133	60	2.22
Grape	*Vitis aestivalis*	Vitaceae	32	35	0.91
Dogwood	*Cornus stolonifera*	Cornaceae	15	25	0.60
Lilac	*Syringa vulgaris*	Oleaceae	21	37	0.57
Hackberry	*Celtis occidentalis*	Celtideae	41	25	1.64

phosphate buffer at pH 8.3 for glycolate and pH 7.6 for lactate. The salt-precipitated enzyme was inhibited 55 % by 0.001 M *p*-chloromercuribenzoate only if the enzyme preparation was first dialyzed. The negligible inhibition of non-dialyzed preparations indicated that dialysis removes a sulfhydryl-group protector from tobacco juice; this may explain why the dialyzed preparation is less stable than the non-dialyzed one. The enzyme in barley juice resembles the dialyzed salt-precipitated enzyme from tobacco, as it loses activity quickly even though stored at a low temperature. It seemed probable that a sulfhydryl protector was absent in barley juice, so barley was chosen as the source of glycolic acid oxidase for the study of sulfhydryl-group inhibition. The data obtained are summarized in table II.

The rates of oxidation of glycolic and lactic acids by barley juice were increased 44 % and 150 %, respectively, by 0.001 M Na_2S. Boiled barley juice plus Na_2S oxidized neither glycolic acid nor lactic acid.

The inhibitory effects of phenylmercuric nitrate and of *p*-chloromercuribenzoate were tested on purified spinach enzyme in the presence of tris-hydroxymethylaminomethane at pH 8.3 for glycolate and pH 7.6 for lactate oxidation. The data obtained are summarized in table III.

LIGHT ACTIVATION OF THE ENZYME WHICH OXIDIZES GLYCOLIC ACID: Tolbert and Burris (11) reported that the enzyme which oxidizes glycolic acid is activated by light. The enzyme was absent from etiolated plants, but was present in the green parts of normal plants. The activation of the enzyme in etiolated barley plants upon exposure to light did not parallel the formation of chlorophyll, for the formation of chlorophyll was relatively slow during the first 6 hours of illumination, the period in which the activation of the enzyme was most rapid. Light had no effect upon the enzyme after its removal from the intact leaves.

In a reinvestigation of the light activation of glycolic acid oxidase, juice expressed from the leaves of etiolated barley consistently oxidized glycolic acid. The rates of oxidation were greater than reported earlier (11) but were much lower than those obtained

TABLE II

INHIBITION OF GLYCOLIC ACID OXIDASE IN BARLEY JUICE BY SULFHYDRYL-GROUP INHIBITORS

SUBSTRATE	PERCENT INHIBITION								
	IODOACETATE		IODOACETAMIDE		PHENYLMERCURIC NITRATE		*p*-CHLOROMERCURIBENZOATE		
	0.001 M	0.01 M	0.001 M	0.01 M	0.0001 M	0.001 M	0.0001 M	0.001 M	0.01 M
Glycolate	0	46	0	20	5	81	4	64	100
DL-lactate	0	30	0	0	28	50	0	60	70*

* Inhibitor partly precipitated from solution at this concentration and pH.

TABLE III

INHIBITION OF GLYCOLIC ACID OXIDASE IN PURIFIED SPINACH ENZYME BY SULFHYDRYL-GROUP INHIBITORS

SUBSTRATE	PERCENT INHIBITION		
	PHENYLMERCURIC NITRATE	*p*-CHLOROMERCURIBENZOATE	
	0.001 M	0.001 M	0.005 M
Glycolate	74	11	41
Lactate	47	18	87

with preparations from green leaves. For example, the $Q_{O_2}(N)$ on glycolic acid for preparations from 5- to 7-day-old etiolated barley plants ranged from 30 to 145, whereas preparations from normal young barley have a $Q_{O_2}(N)$ of about 300. Similarly, plant juice from 7-day-old etiolated garden beans had a $Q_{O_2}(N)$ of 9, while the plant juice from normal young garden beans had a $Q_{O_2}(N)$ of 77. The plant juice from 10-day-old etiolated corn had a $Q_{O_2}(N)$ of 10; plant juice from normal young corn had a $Q_{O_2}(N)$ of 110. These data indicate that etiolated plants contain glycolic acid oxidase, but that its concentration is much less than in normal green plants.

The effect of the age of the etiolated barley plants upon the glycolic acid oxidase activity was tested. Barley was planted on 3 successive days, and the glycolic acid oxidase activity, determined manometrically for 3-, 4-, and 5-day-old etiolated barley, showed $Q_{O_2}(N)$ values of 0, 27, and 30, respectively.

DISCUSSION

The occurrence of glycolic acid oxidase in 14 plant families tested here and 2 families (Solanaceae and Gramineae) tested earlier (4) suggests its importance in the economy of higher plants. In addition to possible functions in photosynthesis (9, 11), Zelitch (20) has pointed out how the enzyme can serve in a terminal oxidation system. It is difficult to assess the importance of these various functions, but, in view of the high activity and wide distribution of the enzyme, it is inadmissible to ignore its potentialities in plant metabolism.

Sulfhydryl inhibitors indicated that glycolic acid oxidase requires -SH groups for enzymatic activity. Iodoacetate, iodoacetamide, phenylmercuric nitrate, and *p*-chloromercuribenzoate inhibit oxidation of glycolate and lactate by the crude barley juice preparations and the two mercurials also inhibit the purified spinach enzyme. The use of sulfhydryl group inhibitors has been discussed in excellent reviews by Olcott and Fraenkel-Conrat (7) and by Barron (1).

Tolbert and Burris (11) reported that the enzyme oxidizing glycolic acid is light activated, for the enzyme was absent from etiolated plants, but was formed upon illumination of the etiolated plants. Experiments on 5- to 7-day-old etiolated barley seedlings failed to confirm the absence of glycolic acid oxidase, although its activity was sharply limited. All preparations from etiolated barley consistently showed significant amounts of glycolic acid oxidase, and limited activity was found in etiolated corn seedlings and etiolated bean seedlings as well. The activity, however, always was much less than in the normal green seedlings. Tolbert, Cohan, and Hendricks (14) have more recently reported that etiolated barley preparations do not consistently lack the ability to oxidize glycolic acid. They have suggested that the increase in activity when etiolated plants are illuminated may result from adaptive enzyme formation in response to glycolic acid formation in the light, for glycolic acid is an early product of photosynthesis (2).

The possibility that the presence of active glycolic acid oxidase may depend upon the age and physiological development of the etiolated seedling was investigated, and it was found that the activity increases from a $Q_{O_2}(N)$ of 0 in 3-day-old, to 30 in 5-day-old barley. This may partially explain the discrepancies observed in the activities of etiolated plants.

SUMMARY

Enzymatic oxidation of glycolic and lactic acids was observed with crude juice from the leaves of all plants tested except for 3 plants from which the juice was difficult to extract. Glycolic acid oxidase activity has been found in a total of 16 common plant families.

Inhibition of glycolic acid oxidase by iodoacetate, iodoacetamide, phenylmercuric nitrate, and *p*-chloromercuribenzoate constitutes evidence that the enzyme requires sulfhydryl groups for its activity.

The etiolated plants tested were not devoid of glycolic acid oxidase, but their enzymatic activity was greatly enhanced by illumination.

The authors wish to acknowledge the generosity of Drs. Zelitch and Ochoa for supplying them with a sample of the purified enzyme from spinach.

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THE OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE BY AN ASCORBATE SYSTEM FROM CUCUMBER[1]

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In recent years there have appeared several contributions (3, 5, 8, 9, 12) bearing on the problem of linkages between H-donating systems on the one hand and ascorbic acid and its oxidase on the other. Thus the demonstration of a sequence of reactions linking the substrate of a triphosphopyridine nucleotide (TPN)-reducing dehydrogenase to ascorbic acid has been made possible by the discovery of a glutathione reductase (3, 8). Another ascorbate system has been described (9) in which the oxidation of reduced diphosphopyridine nucleotide (DPN) was brought about by preparations from peas in apparently cyanide resistant reactions involving ascorbic acid but not ascorbic oxidase.

This paper describes a system present in crude extracts of cucumber which is capable of transferring H from DPNH to the product of ascorbic acid oxidation by its oxidase. Apparently similar systems are under investigation elsewhere (Dr. A. Nason, personal communication and Dr. E. Racker, personal communication). Evidence from manometric experiments with preparations from plants in which substrates for dehydrogenases present in the extracts were oxidised by the ascorbate oxidation system has already indicated that such a linkage exists (5, 6, 7, 12); the present report deals chiefly with some pertinent spectrophotometric data on DPNH oxidation.

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It is realized at the outset that the demonstration of chains of electron- or H-transfers in experiments in vitro only offer possibilities for in vivo mechanisms; they do not allow an answer to the question as to how much of the respiratory H-transfer actually occurs through such a pathway.

MATERIALS AND METHODS

Plant material was obtained locally and frozen at −12°C overnight. It was then allowed to thaw and was ground in a pestle and mortar. The liquid obtained by squeezing the mash through cloth was centrifuged at approximately 6,000 × g for a few minutes and was used without further treatment. The dry weight of such a preparation was 25.4 mg/ml and the protein content, by the Warburg and Christian (13) method was 2.4 mg/ml. Reagents and coenzymes were obtained from commercial sources. DPNH was prepared from DPN (Cozymase "90" Sigma) either chemically by the method of Brodie (2) or enzymatically by adding alcohol and alcohol dehydrogenase. Oxidation of the reduced coenzyme was followed by measuring decrease in absorbance at 340 mμ on a Beckman B spectrophotometer, using cells of 1 cm cross section. The cells usually contained 0.5 ml M/15 phosphate buffer, pH 7.0, 0.1 to 0.3 ml cucumber extract, 0.5 ml (approx. 0.5 mg) DPN or DPNH, 0.2 ml M/25 ascorbate, and water to a total volume of 3.0 ml. Ascorbic oxidase activity of the extracts

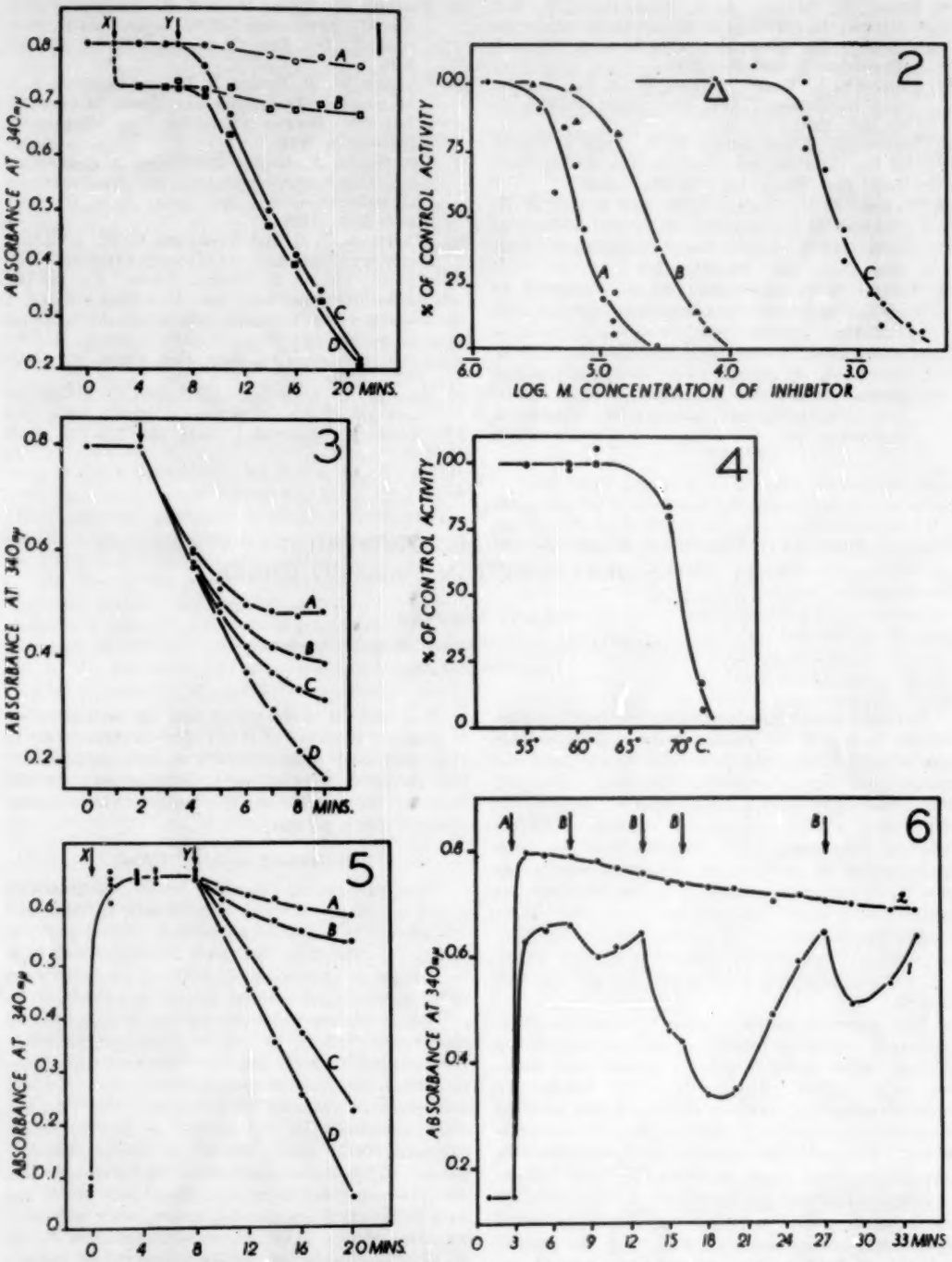

FIG. 1. Oxidation of DPNH by cucumber extract and ascorbic acid. Each of four cells, A, B, C, D, contained buffer and DPNH. At X, ascorbic acid was added to a concentration of 0.0006 M in C and to 0.006 M in D. A and B received corresponding amounts of water. At Y, 0.2 ml cucumber extract was added to all cells. No correction has been made for the dilution resulting from the additions to B and D at X.

TABLE I

THE ABILITIES OF VARIOUS EXTRACTS TO OXIDISE DPNH IN THE PRESENCE AND ABSENCE OF ASCORBATE, AND TO OXIDISE ASCORBIC ACID

	DPNH OXIDATION *		ASCORBIC ACID OXIDATION **
	NO ASCORBATE	PLUS ASCORBATE	
			$\mu l\ O_2$-uptake
Cucumber (fruit)	0.02	0.52	76
Crookneck squash (fruit)	0.00	0.15	81
Acorn squash (fruit)	0.68	0.06	59
Summer squash (fruit)	0.20	0.01	16
Zucchini squash (fruit)	0.02	0.10	16
Barley (etiolated coleoptiles)	0.08	0.22	37
Wheat (etiolated coleoptiles)	0.12	0.12	14
Pea (leaves)	0.06	0.14	45

* Each cell contained 0.2 ml of extract. The figures are the decrease in absorbance, at 340 mμ, in the presence of c 0.5 mg DPNH in the 4 minute period after adding 0.2 ml water or 0.2 ml M/25 ascorbate.

** The figures are μl O_2-uptake induced in 15 minutes by 0.5 ml of the extracts, on adding 0.5 ml M/25 ascorbate. All of the extracts had been stored for 20 hours at 2°C before the above determinations of activity were made.

was measured manometrically in the presence of 0.5 ml M/25 ascorbate.

RESULTS

The addition of either ascorbate or cucumber extract to DPNH did not induce oxidation, but when both were added a rapid oxidation was observed. Figure 1 shows the results of an experiment in which sufficient ascorbate to give final concentrations of 0.0006 M and 0.006 M, respectively, was added, at X, to cells C and D containing DPNH. The absorbance remained constant and indistinguishable from that of control cells (A and B), which received water instead of ascorbate, until cucumber extract was added, at Y. At this point oxidation occurred with equal rapidity in each of the two cells (C and D) to which ascorbate had been added previously. The addition of cucumber extract to the cells without ascorbate induced only a very slow oxidation of DPNH.

TABLE II

THE EFFECTS OF VARIOUS TREATMENTS ON THE ABILITY OF THE CUCUMBER SYSTEM TO OXIDISE DPNH

TREATMENT	CHANGE IN ABSORBANCE AT 340 Mμ IN 3 MINUTES AS % OF CHANGE IN CONTROL
1) Control *	100
2) Extract dialysed for 24 hours	36
3) As 2 with concentrated dialysate	37
4) Extract dialysed for 48 hours	10
5) 0.001 M iodoacetate	97
6) 0.0001 M diethyldithiocarbamate	8
7) 0.0001 M KCN	53
8) Crystalline catalase added	100
9) Cucumber extract omitted	0
10) Cucumber extract boiled	0
11) Dehydroascorbic acid in place of ascorbic acid	5
12) TPNH in place of DPNH	0

* The control system contained DPNH, cucumber extract, and ascorbate added at zero time.

Comparable data from other plant materials is shown in table I. It will be seen that four of the extracts reacted in the same manner as cucumber, though less effectively. Several of the extracts, in contrast to that from cucumber, induced a rapid oxidation of DPNH without benefit of ascorbate addition, and, indeed, in some of these the addition retarded this spontaneous oxidation. An important point is that the ability to oxidise DPNH was not a direct function of the ability to oxidise ascorbic acid, since only a few of the extracts utilized ascorbate in the DPNH oxidation whereas all were capable of oxidising ascorbate itself. It is conceivable that purification steps may reveal that others of the extracts contain a similar DPNH oxidation system to that from cucumber; however, for the present purposes, the selection for further work of cucumber, whose extracts show a very low "blank" oxidation of DPNH and a striking response to added ascorbate without further treatment, was an obvious one.

FIG. 2. The effects of 2,3-dimercaptopropanol on: A, the oxidation of DPNH by the cucumber-ascorbate system, and C, the oxidation of ascorbic acid by the oxidase. Curve B shows the effects of *p*-chloromercuribenzoic acid on the DPNH oxidation, and the open triangle at 10^{-4} M shows the effect on ascorbic acid oxidation.

FIG. 3. The effects of 2,3-dimercaptopropanol on DPNH oxidation. The cells contained cucumber extract and DPNH, with 2,3-dimercaptopropanol in the following concentrations: A, 10.4×10^{-6} M; B, 7.8×10^{-6} M; C, 4.5×10^{-6} M; and D, 0. 0.006 M ascorbate was added to all cells at the time indicated by the arrow.

FIG. 4. The effect of 1 minute's pretreatment at different temperatures on the subsequent DPNH oxidation (open circles), and on ascorbic oxidase activity (closed circles).

FIG. 5. Competition between the DPNH oxidation system and alcohol dehydrogenase plus alcohol. Cells A, B, C, D, contained DPN and cucumber extract, with alcohol dehydrogenase in the following amounts: A, 1.0 ml; B, 0.3 ml; C, 0.1 ml; D, 0.02 ml. At X, alcohol (0.1 ml) and, at Y, ascorbate (0.006 M) were added to all cells.

FIG. 6. The effects of added ascorbate and of aeration on the status of the coenzyme in the presence of cucumber extract and an H-donating system. Each of the two cells contained cucumber extract, alcohol dehydrogenase, DPN, and buffer, and one cell (lower curve) contained ascorbate (0.006 M) in addition. At A, 0.1 ml alcohol was added to both cells and at the points, B, they were briefly shaken.

The ascorbic oxidase in the cucumber extracts was shown not to be inactivated during storage at 0°C for several days. The ability of the extracts to oxidise DPNH, however, declined after the first 24 hours of storage. As shown in table II there was also a considerable loss in activity (which was not reversed by adding concentrated dialysate) when the cucumber extracts were dialysed, but similar treatment was without effect on the ascorbic oxidase. It was thus clear that the cucumber extracts contained a component other than ascorbic oxidase which was essential for the oxidation of DPNH and this observation was apparently confirmed by experiments in which a sample of commercially prepared dehydroascorbic acid (DHA) alone failed to induce oxidation of DPNH. However, when an equivalent amount of DHA was added instead of ascorbate at pH 6.6 to the cucumber extracts in the presence of DPNH, the unexpected result was obtained that the coenzyme was not oxidised. The subsequent rapid oxidation of the DPNH which occurred when ascorbate was added to the system showed that the DHA did not contain inhibitory materials, and the failure of the extracts to use it as oxidant would indicate that some other product of ascorbic acid oxidation by the enzyme is the actual H-acceptor (10).

The Effects of Inhibitors on the Oxidation of DPNH: The addition of inhibitors of ascorbic oxidase were strikingly effective in preventing or slowing down the oxidation of DPNH. Cyanide, and particularly diethyldithiocarbamate were inhibitory at 10^{-4} M (table II). Iodoacetate was without effect at 10^{-3} M and only slightly inhibitory (7 %) at 3×10^{-3} M, but *p*-chloromercuribenzoic acid, at concentrations as low as 10^{-4} M, which had no effect on the ascorbic oxidase component of the extracts, prevented DPNH oxidation completely (fig 2, curve B).

A similar differentiating effect was shown when BAL (2,3-dimercaptopropanol) was added. The ascorbic oxidase is itself affected by this inhibitor but only when concentrations of 3×10^{-4} M are exceeded (fig 2, curve C). The ability to oxidise DPNH, on the other hand, was virtually completely lost at concentrations some 50 times lower than this (curve A).

When the concentrations of BAL inducing equal degrees of inhibition of a) the ascorbic oxidase, and b) the oxidation of DPNH are compared, it is seen that the former are about one hundred times the latter. Figure 3 shows the time course of DPNH oxidation when ascorbate is added to extracts in the presence of different concentrations of BAL. It is seen that in the first 2 minutes of the reaction the rates of oxidation in the presence of the inhibitor are not very different from that of the control. Thereafter the curves diverge sharply; the rates of oxidation declined in those cells containing inhibitor and, in the presence of the highest concentration of BAL, the oxidation came to a virtual halt. For a probable explanation of the delay in the onset of inhibition see Slater (11). In figure 2 the BAL effects on the oxidation of DPNH are those apparent during the period 3 to 7 minutes after addition of ascorbate; the figures for BAL inhibition of the ascorbic oxidase are effects observed during the 15 minute period begun by adding the substrate.

The effects of heat on the system are shown in figure 4. The ability to oxidise DPNH is completely lost after one minute at 70°C but since the response of the DPNH oxidation system is precisely that of the ascorbic oxidase, it may be presumed that the sensitivity of the overall system is determined by that of the oxidase.

Linkage with an H-donating System: Figures 5 and 6 show results obtained when alcohol dehydrogenase (diluted from a crystalline product) and alcohol were used to generate the reduced coenzyme, in the presence of cucumber extracts. In figure 5 four levels of alcohol dehydrogenase were employed and the DPN was reduced with equal alacrity in all cases. The subsequent addition of ascorbate to each cell resulted in oxidation of the DPNH but at widely different rates. Curve D, which was obtained from the cell containing the smallest amount of dehydrogenase, showed a sharp linear decline, but proportionately slower oxidation was recorded when larger amounts of the dehydrogenase were present (curves C, B, A). In curve A, the activity of the reducing system was apparently sufficiently high to maintain a high percentage of the coenzyme in the reduced condition, whereas, in the presence of decreasing amounts of the dehydrogenase the oxidation system competed with increasing effectiveness.

Figure 6 shows the results of an experiment in which alcohol was added, at A, to systems containing alcohol dehydrogenase, cucumber extract, and DPN, in the presence (1) and absence (2) of ascorbate. There was a rapid reduction of DPN in each case but in the absence of ascorbate this was more complete. Subsequently both systems were shaken briefly at the times indicated by the arrows, B. Immediately after each such aeration a decrease in absorption, indicating oxidation of DPNH, was observed in the presence of ascorbate, whereas, in its absence (curve 2) the absorption declined slowly and steadily. The oxidation of DPNH which occurred in (1) was succeeded by reduction when the cells were allowed to remain unshaken. It appears, then, that the status of the DPN in the system can be altered towards oxidation by facilitating the oxidation of ascorbate by the oxidase in the extracts, or towards reduction by allowing the reductive process controlled by the dehydrogenase to continue unchecked. One can with some reason conclude that the ascorbic system coupled, by components in the extracts, to DPNH and to the dehydrogenase is capable of oxidising the substrate alcohol, at the ultimate expense of atmospheric oxygen. Manometric experiments in which ascorbate was added to cucumber extracts with alcohol dehydrogenase, alcohol, and DPN have in fact shown that O_2-uptake is slightly increased (by 10 to 20 μl) over controls without added alcohol. However, spectrophotometric examination of the reaction mixtures after such trials revealed that the shaking in the manometer in the presence of alcohol reduces the ability of the system

to oxidise DPNH, although the ascorbic oxidase activity is undiminished by such treatment. No successful means of overcoming this fragility of the oxidation system has yet been found.

Discussion

Several features of the DPNH oxidation system in cucumber extracts may be mentioned which distinguish it from other systems which have already been described. Since TPNH cannot replace DPNH as H-donor the operation of the glutathione reductase sequence present in peas (8), wheat (3), and a number of other tissues including cucumber (1) can be ruled out in this case. Further, it was shown that no reduction of glutathione (GS-SG) or of cystine was induced by the cucumber extracts in the presence of DPNH, and the participation of these H-acceptors may therefore be discounted. The essentiality of the ascorbic oxidase distinguishes the cucumber sequence from a DPNH oxidation system described from peas (9) and the lack of inhibition by catalase and the essentiality of ascorbic acid would discount the possibility of the operation of a peroxidase-containing system analogous to the TPNH oxidase described by Conn et al (4).

An essential component for the oxidation of DPNH by the ascorbate system was shown to be extremely sensitive to the -SH reagents, 2,3-dimercaptopropanol and *p*-chloromercuribenzoic acid; complete inhibition was obtained at concentrations which had no effect on the ascorbic oxidase. On the other hand, the system was stable towards 10^{-3} M iodoacetate. It appears likely that this component is instrumental in facilitating the transfer of H from DPNH, since Nason (10) has recently purified such an enzyme from peas. As in the case of cucumber, the H-acceptor was shown not to be dehydroascorbic acid, although ascorbic acid and its oxidase afforded an H-accepting system. Mathews (9) also showed that dehydroascorbic acid did not function as H-acceptor in his (cyanide stable) system and suggested that monodehydroascorbic acid may be the actual H-acceptor. The evidence is accumulating to suggest that in those sequences of DPNH oxidation involving ascorbic oxidase also, such a compound may be the penultimate H-acceptor in the transfer of electrons from DPNH to atmospheric oxygen.

Summary

Crude extracts from cucumber have been shown to induce a rapid, cyanide sensitive oxidation of DPNH when ascorbic acid is added in small amounts. The oxidation system involves ascorbic oxidase present in the extracts and another component which is extremely sensitive to 2,3-dimercaptopropanol and *p*-chloromercuribenzoic acid. Some other properties of the oxidation mechanism are described. In spectrophotometric experiments the ascorbate-induced DPNH oxidation has been linked to an H-donating system consisting of alcohol and alcohol dehydrogenase.

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THE PROTEINS OF GREEN LEAVES. VI. CENTRIFUGAL FRACTIONATION OF TOBACCO LEAF HOMOGENATES AND SOME PROPERTIES OF ISOLATED CHLOROPLASTS[1,2]

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Chloroplasts of higher plants have been isolated from leaf homogenates, with greater or lesser attempts at purification from other types of particles, using differential centrifugation. Such preparations of chloroplasts, or "chloroplastic matter" (see earlier reviews, 27, 36), have in some cases been reported to contain a series of components; for instance nucleic acid (10, 15, 26), catalase (23, 24), or cytochrome oxidase (11, 12, 28, 31). Critical proof of the presence of these in the chloroplasts, however, requires the preparation of plastids freed from all other types of subcellular particles. We have accordingly investigated the process of purifying tobacco leaf chloroplasts, and have been able to obtain intact whole chloroplasts evidently free from most other leaf components. These plastid preparations had negligible amounts of the two enzymes mentioned above, and had very little nucleic acid.

Our preparation of purified chloroplasts has enabled us to study the non-plastid particles of tobacco leaf homogenates. All other centrifugal fractions are contaminated with chloroplasts or chloroplast fragments, in our experience. Having purified chloroplasts, it becomes possible to estimate the contribution of the chloroplasts to each successive fraction, and the properties of the non-plastid particles are found by difference.

As a result of our studies, we are able to point out a number of complications to the problem of separating subcellular organelles from leaf tissue. With full recognition of these complications, we have been able to devise methods for analyzing a leaf homogenate for four different classes of particles, as well as for purifying chloroplasts.

MATERIALS AND METHODS

Turkish tobacco plants (*Nicotiana tabacum* var. Samsun) were grown in 6-inch pots in the greenhouse. The plants were kept in a darkened room for 1 to 3 days in order to remove starch grains from the chloroplasts; mature leaves were then detached and the petioles and midribs removed. The leaf tissue was ground in the cold in 4 to 5 times its weight of cold M/15 phosphate buffer at pH 7.0 made to 0.4 M sucrose. Leaves were homogenized either by grinding with a mortar and pestle, or else by a 30-second to 1-minute blending at 55 % of line voltage in an Omnimixer. The homogenates were strained through a pad of cheesecloth plus glass wool to remove cell walls, or occasionally through sharkskin filter paper using a basket centrifuge. Centrifuged pellets were made up to a known fraction of the volume of the strained homogenate, and measurements of the suspensions were calculated back to ml homogenate.

To obtain particles from these homogenates, 40-ml aliquots were centrifuged for 12 minutes at selected speeds. The relative centrifugal force (RCF) was calculated for the mid-point of the fluid volume in the centrifuge tube at each speed used. Pellets were resuspended and chloroplasts and any other particles were washed in the same sucrose-phosphate buffer. All operations were performed in a cold room at 4°C.

Protein nitrogen (N) was measured in resuspended pellets or in soluble supernatants after precipitation with cold 1 *N* trichloroacetic acid (TCA) and one wash with cold *N*/2 TCA. The precipitates were digested with sulfuric acid using a selenium, mercuric sulfate, potassium sulfate mixture as catalyst, and N after digestion was measured by Nesslerization. Phosphorus (P) measurements were made on perchloric acid digested material, according to the method of Berenblum and Chain (4). Total chlorophyll determinations were carried out by measuring absorption at 645 and 663 mμ in acetone extracts, according to the procedure described by Arnon (3). Iron was measured by the method of Sayvell and Cunningham (29). Hill reaction activity was measured by following the reduction of ferricyanide potentiometrically, by a modification of the method of Spikes et al (32).

Nucleic acid components were extracted following the procedures developed by Ogur and Rosen (25) for use with plant material. The methods call for preliminary extractions to remove interfering materials, then selective extraction of the ribose nucleic acid (RNA) components by 1 *N* perchloric acid at 4°C, and final extraction of the desoxyribose nucleic acid (DNA) components in *N*/2 perchloric acid at 70°C. For routine analyses, the cold acid extraction was omitted, and the total nucleic acid was extracted at 70°C. These nucleic acid extracts were analyzed for P, ultraviolet absorption curve, and for desoxyribose with the Dische diphenylamine test (8). RNA in the extracts was then estimated as the difference between total nucleic acid found from absorption at 260 mμ and phosphorus values, and the DNA present measured by the diphenylamine test. Pentose analyses by the orcinol method were found, in many extracts, to give values up to ten times higher than the phosphorus or ultraviolet analyses would indicate.

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These high values were assumed to be due to interfering materials, and were not recorded.

Catalase determinations were made in the apparatus described by Appleman (2) in which the final H_2O_2 concentration was 0.6 N and the tests were performed at 0°C. Cytochrome oxidase was determined manometrically at 25°C using two flasks, one with 3 mg hydroquinone, and the other with hydroquinone plus 7×10^{-5} M cytochrome c. Final volume in the flasks was 2.5 ml. Cytochrome oxidase activity was measured as the difference in O_2 uptake between the two flasks. All enzyme determinations were performed in duplicate.

Experimental Results

Sedimentation Behavior of Desoxyribosenucleic Acid: By following desoxyribose nucleic acid (DNA) distribution in successive centrifugations of a leaf homogenate, it is possible to gain an idea as to the behavior of the nuclei. The assumption is made that under our conditions the bulk of the DNA of the cell is localized in the nucleus. This seems likely even considering the work of Chayen (6), who found DNA in cytoplasmic particles, but only in meristematic cells which were entirely undisturbed.

Our first experiments used homogenates strained through sharkskin filter paper in a basket centrifuge. There was great difficulty in finding enough DNA to measure in these experiments. We later found that there is a very marked and selective retention of DNA on sharkskin filter paper. In table I, DNA and protein N concentrations are compared in separate aliquots of a homogenate strained through sharkskin and through glass wool, respectively. Aliquots filtered through sharkskin filter paper can be seen to have lost 70 to 85 % of their DNA, but only 10 to 12 % of the total protein N, compared to aliquots filtered through glass wool. This retention by sharkskin filter paper has been used in removing nuclei from a given preparation of chloroplasts.

The distribution of DNA when homogenates are centrifuged indicates that by far the largest part of the DNA is associated with particles, most likely nuclei, sedimenting at low speeds. In figure 1 the total DNA brought down as a sediment in 12 minutes is plotted as a function of the RCF used in successive centrifugations of a single aliquot from one homogenate. There is a large difference between homogenates prepared by Omnimixer blending, and those prepared by mortar and pestle grinding. Mortar and pestle grinding leaves the nuclei sedimentable at a lower RCF, and evidently intact. The brief Omnimixer treatment alters the nuclei so that they are not fully sedimented until about $600 \times g$; and only 50 % sedimented at the force of $76 \times g$, which brings down 95 % of the nuclei from homogenate prepared by mortar and pestle.

Table I

Retention of Homogenate DNA by Sharkskin Filter Paper

Grinding procedure	Straining procedure	Homogenate protein N	Loss on paper	Homogenate DNA-P	Loss on paper
		mg	%	*mg*	%
Mortar and pestle	Cheesecloth and glass wool	48.0	12	0.044	82
	Filter paper	43.4		0.008	
Omnimixer	Cheesecloth and glass wool	42.2	11	0.037	68
	Filter paper	37.4		0.012	

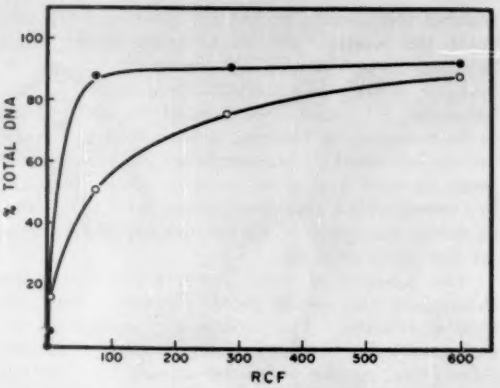

Fig. 1. The sedimentation of DNA from a tobacco leaf homogenate. The DNA sedimented is plotted as a function of the RCF used to bring each successive pellet down. The upper curve shows DNA sedimentation from a homogenate prepared by grinding leaves with a mortar and pestle; the lower one from a homogenate resulting from grinding leaves with an Omnimixer. The sedimentation is virtually complete in the first homogenate at $76 \times g$; in the second case not until $600 \times g$.

Chlorophyll Sedimentation: In order to purify chloroplasts centrifugally, it seemed advisable to study the sedimentation behavior of the plastids. This was done by measuring the chlorophyll content of a series of centrifugal pellets obtained from a leaf homogenate. In figure 2 the chlorophyll brought down in 12 minutes is plotted as a function of the RCF employed. RCF values are plotted on a logarithmic scale merely to put the entire range of forces on one graph. In homogenates prepared by Omnimixer grinding of the leaves, 90 % of the chlorophyll is sedimented by an average RCF of $650 \times g$ in 12 minutes. Microscopic examination of the green pellets brought down at this force shows that the chlorophyll is contained in chloroplasts which appear whole and intact. By contrast, mortar and pestle grinding of the leaves, with or without sand, causes disruption of the chloroplasts so that only 50 % of the chlorophyll is sedimented at $650 \times g$. The remainder of the plastids are in the form of fragments which can only be centrifuged down when forces up to $10{,}000 \times g$ are applied.

Chloroplasts are thus seen to respond in an entirely different manner from nuclei to the two homo-

genizing treatments; mortar and pestle grinding disrupts the plastids and leaves nuclei intact, while blending disrupts nuclei, but leaves the plastids essentially intact. Mitochondria from animal tissues, like nuclei but unlike these chloroplasts, are reported to be disrupted by blending (especially if intensive), but are left intact by homogenizing with a mortar and pestle or with a glass homogenizer (30). Grinding leaf tissues with a glass homogenizer is found to cause as serious disruption of the chloroplasts as does mortar and pestle grinding.

The presence of large starch grains inside the chloroplasts also causes plastid disruption with any type of grinding. This is especially evident on resuspension and subsequent centrifugations of the chloroplasts, as the grains are apparently torn out of the plastid during handling. For this reason all plants were kept in the dark before the leaves were harvested.

The green pellets obtained from Omnimixer homogenates at 650 × g can be observed to have other components besides chloroplasts. Nuclei (mostly elongated), smaller particles, and, as yet, unidentified crystals are all seen to be present. Nuclei and a large part of the crystals can be removed partly by low speed centrifugations, and partly by repeated passage through sharkskin filter paper. The smaller, non-green particles can be removed by repeated centrifugal washings. Using the curve shown in figure 2 it is possible to pick a centrifugal force for the washings which will sediment a major portion of the intact plastids. At 500 to 650 × g for 12 minutes, ¾ or more of the intact tobacco chloroplasts will be sedimented, and the maximum possible separation of plastids from the smaller particles is achieved. By repeating this process 5 to 8 times, we have been able to obtain whole chloroplasts, visibly free of the smaller particles.

PROTEIN NITROGEN DISTRIBUTION IN LEAF HOMOGENATES: After 90 % of the chloroplasts are removed at 650 × g, the pellets that can be brought down at higher speeds are still green, but have a lower chlorophyll content per mg N. As shown by the data in table III, the chlorophyll concentration of successive pellets goes from 0.40 to 0.65 to 0.36 to 0.13 mg chlorophyll per mg N in a typical homogenate. On the other hand, the chlorophyll content of our washed chloroplast preparation is most frequently found to be close to 1.0 mg chlorophyll per mg N. After isolation and purification, such washed chloroplasts can be

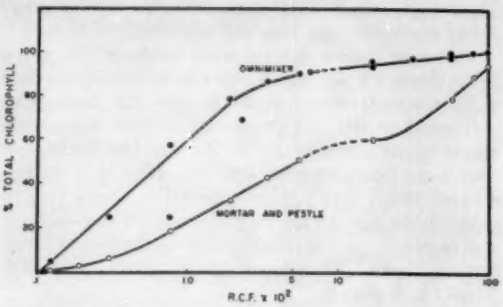

FIG. 2. The sedimentation of chlorophyll from tobacco leaf homogenates. Chlorophyll sedimented is plotted as a function of the RCF used to bring each successive pellet down. RCF is on a log scale in order to include all forces used. The points on the upper curve are taken from two separate experiments in which leaves were ground using an Omnimixer; those in the lower curve from one experiment using leaves ground in a mortar and pestle. Sedimentation is 90 % complete in the first case at 650 × g; in the second case not until 10,000 × g. The first half of the curve is from pellets brought down in an International Clinical centrifuge; the points on the last half of the curve are from pellets sedimented in a high speed Servall centrifuge.

TABLE II

DISTRIBUTION OF PROTEIN NITROGEN IN A TOBACCO LEAF HOMOGENATE *

FRACTION	CHLOROPHYLL	CHLOROPLAST N	PROTEIN N	NON-CHLOROPLAST N	PROTEIN N AS % TOTAL PROTEIN N IN HOM.	NON-CHLOROPLAST N AS % TOTAL PROTEIN N IN HOM.
	MG PER ML ORIGINAL HOMOGENATE				%	%
Original homogenate ..	0.155	0.155	0.475	0.320	100	68
100 × g sed.**	0.054	0.054	0.070	0.016	14.7	3.2
650 × g sed.**	0.083	0.083	0.108	0.025	22.7	5.3
8,000 × g sed.**	0.018	0.018	0.045	0.028	9.5	5.9
22,000 × g sed.†	0.004	0.004	0.023	0.019	4.8	4.0
Total particulate	0.159	0.159	0.246	0.082	51.7	18.4
Supernatant	....	....	0.220	0.220	46.3	46.3
Recovery	0.159	0.159	0.466	0.302	98.0	64.7

* Homogenate prepared by blending, then straining through cheesecloth and glass wool.
** 12 minute sedimentation.
† 45 minute sedimentation.

TABLE III

SEDIMENTATION OF PARTICULATE CATALASE, PROTEIN NITROGEN, AND CHLOROPHYLL FROM A TOBACCO LEAF HOMOGENATE*

FRACTION	CHLOROPHYLL	CHLOROPHYLL/MG N	CATALASE	CATALASE/MG N	CATALASE/MG NON-CHLOROPLAST N
	% OF HOM.		% OF TOTAL IN PARTICLES	ML O_2/SEC	ML O_2/SEC
Original homogenate ...	100	0.28	..	0.18	0.25
30×g— 7 min.	4	0.40	8	0.10	0.16
500×g— 7 min.	77	0.65	27	0.05	0.13
4,000×g—12 min.	12	0.36	46	0.38	0.61
22,000×g—30 min.	3	0.13	19	0.18	0.21
Purified chloroplasts	...	1.0	..	0.002	...

* Homogenate prepared by blending, then straining through cheesecloth and glass wool.

artificially fragmented and the smaller fragments separated from the remaining intact plastids. Analysis of the fragments always shows that they have the same chlorophyll/N ratio as the original preparation of intact plastids. Therefore, the lower chlorophyll/N ratios in successive pellets brought down from the whole tobacco leaf homogenates must result from the presence of particles that are neither chloroplasts nor chloroplast fragments.

It is possible to obtain an accurate estimate of the non-plastid particles present, assuming that the chlorophyll in all pellets is associated only with either whole chloroplasts or chloroplast fragments. The total chloroplast N of a particular fraction can be calculated from the total chlorophyll of the fraction, once the chlorophyll/N ratio is known for pure chloroplasts from the same homogenate. Non-plastid N is then calculated as the difference between the total N and the chloroplast N of the fraction. For instance, the fraction sedimenting at 8000×g in table II has 0.018 mg chlorophyll/ml. Since, in this homogenate, purified chloroplasts had a chlorophyll/N ratio of 1.0, that means that the pellet has 0.018 mg chloroplast N/ml. The total N was 0.045 mg/ml, so that the remainder, or 0.028 mg N/ml, must have been non-plastid N. The other fractions of this homogenate are similarly analyzed in table II. It can be seen that 52 % of the protein N of this homogenate is particulate. By chlorophyll analysis of the whole homogenate, 32% of the protein N is in the chloroplasts. The remaining 20 % must be considered to be in non-plastid particles. This non-plastid particle material is distributed through all the pellets; as seen in column 4 of table II (non-chloroplast N/ml homogenate), most of it is in the 650×g and 8,000×g pellets.

In all the homogenates that have been examined, the particles accounted for 45 to 55 % of the total protein. Over half of the particles consisted of chloroplasts, which accounted for 22 to 35 % of the total homogenate protein. Non-plastid particles, then, accounted for the remaining 18 to 25 % of the total homogenate protein. In homogenates prepared by mortar and pestle grinding of the leaves, about 5 % of the total protein could be ascribed to particles sedimenting prior to the chloroplasts. These were presumably nuclei, or what remains of nuclei after their isolation in an aqueous medium. Stern and Mirsky (33) have presented evidence to show that nuclei suspended in aqueous media rapidly lose major amounts of protein and enzymes. Under our conditions, therefore, about 5 % of the total cell protein may be ascribed to residual nuclear material, still sedimenting at a low centrifugal force. This means that 13 to 20 % of the total protein of a homogenate can be accounted for as particles smaller than chloroplasts.

DISTRIBUTION OF CATALASE IN LEAF HOMOGENATES: In making analyses for catalase, it has been our constant observation that 45 to 60 % of the catalase activity of the strained homogenate is not brought down as a sediment in 45 minutes at an RCF of 22,000×g, and at least part of the catalase can be thus considered as part of the soluble protein. This finding is at variance with a number of observations in the literature, where only 10 to 20 % of the total catalase activity of a leaf has been found to be soluble (23, 24), although it does agree with some other observations (13, 16, 20). It is likely that factors not explored in the present experiments, such as ease of solubilization of the enzyme, or precipitation of the enzyme by a low pH of the homogenate (13), might explain these discrepancies.

Our main concern has been with the catalase found in the particles. The distribution of particulate catalase in successive centrifugal pellets is shown in table III, where it can be compared to the centrifugal behavior of chlorophyll and of protein N. The data show that only 35 % of particulate catalase was removed as a sediment when 81 % of the chlorophyll and probably all of the intact plastids were sedimented. Thus, at most 35 % of the particulate catalase might be associated with the chloroplasts. Since the separation achieved in the horizontal centrifuge tubes is incomplete, it remains a distinct possibility that the catalase activity found in the crude chloroplast fraction is still associated with particles smaller than intact plastids. Indeed, on washing such a chloroplast preparation 5 to 8 times by centrifugation at 600×g and resuspension in fresh sucrose buffer, whole and intact chloroplasts are obtained with essen-

tially no catalase activity (table III). The catalase that was removed by washing the first, crude preparation can still be centrifuged out of the supernatant wash solutions. Although full recovery of the catalase of the original crude preparation could not be achieved by centrifuging the wash solutions, still no catalase activity was found in the final supernatants of these wash solutions. These experiments indicate that much of the catalase activity of the first pellet was not present as a protein inside the whole chloroplast and made soluble by later washings, but was rather attached to smaller particles mixed in with the chloroplasts.

Further support for the view that catalase is associated with non-chlorophyll containing particles comes from measurements of catalase concentration (activity per mg protein N) as shown in table III. Concentration of the enzyme is very low in the 560 × g fraction, which contains over 77 % of the chlorophyll, and which has by far the highest concentration of chlorophyll per mg protein N. Catalase activity per mg N rises to a very high level in the next smaller fraction (4,000 × g); the value is 7 times that of the chloroplast-rich fraction, and about twice as high as the original homogenate level in this experiment. The chlorophyll-rich fraction, on the other hand, has catalase activity diluted compared to the original homogenate.

There are two reasons for believing that the chloroplasts in a highly washed preparation, no longer showing catalase activity, are in a reasonably sound physiological condition. When prepared in 0.01 M KCl plus the usual buffer, they retain most or all of the original Hill reaction activity. Thus in one experiment, where the original homogenate was able to reduce 1.42×10^{-7} moles of ferricyanide per mg chlorophyll per minute, the repeatedly washed chloroplasts could reduce 1.40×10^{-7} moles of ferricyanide per mg chlorophyll (Hill reaction) per minute. The plastids were not changed with respect to their activity by removal of the catalase. In addition, these plastids appear morphologically whole and intact. The best evidence for this comes from the fact that, when placed in a hypotonic medium at pH 8, the membranes were seen to rise from the surface of the washed plastids in large blisters, which eventually broke. This swelling process occurred with spinach chloroplasts, or chloroplasts from young tobacco leaves at pH 7, but for some unknown reason did not occur with mature tobacco leaf chloroplasts from plants grown under our conditions, except at a slightly higher pH. Pictures of chloroplasts with raised membranes were published by Weier and Stocking (37). The fact that the membranes were still capable of swelling indicates the existence of an entire, intact membrane. If a hole had been torn in the surface membrane of the plastid, the membrane would not act as an osmotic barrier. The observation of swelling, therefore, rules out the possibility that smaller particles originally inside the chloroplasts broke out through a distinct hole in the membrane during the centrifugations. However, most of the catalase removed from the original chloroplast pellet by washing was still found attached to small particles of sedimentable size. This catalase, therefore, did not originate inside the chloroplasts.

All of these data indicate that tobacco leaf chloroplasts have either no or negligible catalase activity. Rather the enzyme is localized in smaller particles, whose sedimentation by one or two centrifugations under these conditions seriously overlaps that of the chloroplasts. Very similar results were obtained with one spinach leaf homogenate, and purified spinach chloroplasts.

The data of table III show also that the smallest particles that can be centrifuged out of solution by a Servall centrifuge are relatively poor in catalase, providing evidence for a fourth type of particle which can be defined as poor or lacking in catalase, and not completely sedimented until forces up to 22,000 × g are applied for a considerable period of time.

The force at which sedimentation of catalase-rich particles is complete was investigated in experiments such as those shown in table IV. The amount of particulate protein N and particulate catalase remaining in the supernatant after successively faster centrifugations of an original homogenate is listed in this table. Particles to be measured were removed from the supernatant solutions by a force of 22,000 × g, resuspended, and assayed for protein N and catalase. Enzyme activity of these supernatant particles reaches a low and fairly constant level only after a 12-minute centrifugation at 8,000 × g, indicating that this force is required to remove most of the catalase-rich particles from suspension. In the microscope, the particulate fraction rich in catalase is seen to contain, in addition to chloroplast fragments, large numbers of short rods or spheres, from 0.2 to 2 μ in length. These are observed to stain a blue-green color when Janus Green B is added at 0.005 %.

Experiments identical in design with those described above have shown cytochrome oxidase activity to have the same distribution through these centrifugal fractions as does catalase (table V). Activity per mg protein N is very low where the chloroplasts are concentrated, and much higher in the pellet containing

TABLE IV

PARTICLES REMAINING UNSEDIMENTED BY PRELIMINARY 12-MINUTE CENTRIFUGATIONS

PRELIMINARY 12-MINUTE CENTRIFUGATION	SUPERNATANT PARTICULATE PROTEIN N *	CATALASE ACTIVITY IN PARTICLES/MG N
RCF	MG	ML O_2/SEC
3,300 × g	1.05	0.150
8,000 × g	0.86	0.012
10,000 × g	0.60	0.010
12,000 × g	0.53	0.009
14,000 × g	0.56	....
16,500 × g	0.42	0.007

* Brought down by a force of 22,000 × g in 45 minutes.

TABLE V

CYTOCHROME OXIDASE DISTRIBUTION IN A TOBACCO LEAF HOMOGENATE *

FRACTION	TOTAL CHLOROPHYLL	CYTOCHROME OXIDASE/ML HOMOGENATE	TOTAL CYTOCHROME OXIDASE	CYTOCHROME OXIDASE/MG N
	%	ML O_2/MIN	%	ML O_2/MIN
550 × g	74	0.042	23	0.36
8,000 × g	24	0.135	71	2.28
22,000 × g	4	0.010	5	0.88
Purified chloroplasts (several experiments)	..	....	..	0.01–.05

* Homogenate prepared by blending, then straining through cheesecloth and glass wool.

smaller particles. Well washed chloroplasts had negligible cytochrome oxidase activity.

CHEMICAL COMPOSITION OF PURIFIED CHLOROPLASTS: Highly purified chloroplasts have been prepared by repeated washings in sucrose-buffer at 600 × g. Nuclei were eliminated from these preparations, insofar as possible, by three low speed centrifugations and by repeated passage of the chloroplast suspension through sharkskin filter paper. Analyses of the chloroplasts for nitrogen (N), chlorophyll, phosphorus (P), dry matter, iron, and nucleic acid are listed in table VI. Dry weights were determined after 2 further washings with distilled water. The plastids were counted in a bacterial counting chamber, and the analytical results are expressed per chloroplast as well as per mg N.

The analyses for N per plastid were repeated with 9 different preparations, with quite uniform results. The figure for N can be expressed as $6.0 \pm .35 \times 10^{-10}$ mg N per plastid. Values for chlorophyll were somewhat more variable than N, and ranged from 0.8 to 1.0 mg chlorophyll per mg chloroplast N. The reasons for the variation are not clear, but it was a common observation with previous preparations of washed chloroplasts (5, 14, 18), and we think it may be inherent in the plastids and related to the nutritional condition of the leaf (18). Iron and phosphorus values are similar to those previously reported (27), although the iron-containing enzymes, catalase and cytochrome oxidase, were absent from these preparations. It is likely that some iron is present in cytochrome f, according to the data of Davenport and Hill (7).

Attempts were made to determine the nucleic acid content of purified whole chloroplasts by the methods of Ogur and Rosen (23). In spite of preliminary extractions with cold alcohol, boiling alcohol-ether, and briefly with cold perchloric acid, the final, 20 minute, hot $N/2$ perchloric acid extract of the chloroplasts showed only a shoulder at 260 mμ in the otherwise smooth ultraviolet absorption curve, rather than a specific peak at 260 mμ as might have been expected for pure ribose nucleic acid components. However, since the extracts contained organic phosphorus, and produced both the orcinol reaction for ribose (although at too high a level) and the Dische diphenylamine test for desoxyribose, it was possible to estimate the maximum nucleic acid content of whole chloroplasts.

The data in table VI show that the nucleic acid content of washed chloroplasts is very low, since at the very most only 1 % of the total N or 10 % of the total P of washed chloroplasts can be considered as belonging to nucleic acid. Consequently, washed chloroplasts appear to contain from 0.3 to 0.7 % nucleic acid on a dry weight basis.

According to Ogur and Rosen (23), RNA can be preferentially extracted in 1 N perchloric acid in 18 hours at 4°C. DNA, on the other hand, must be subjected to $N/2$ perchloric acid at 70°C, for hydrolysis and solubilization to occur. Application of this test to chloroplasts suggests that about 2/3 of the total nucleic acid present is DNA. The Dische test for desoxyribose indicated the presence of some DNA in the hot acid extracts, but the amounts were so small that it was impossible to say if the nucleic acid present was all accounted for as DNA. Further, a small diphenylamine reaction was found in the cold acid extracts. Consequently, it is not yet possible to decide whether the small amount of nucleic acid present in these preparations is partly or all DNA.

NUCLEIC ACID DISTRIBUTION IN LEAF HOMOGENATES: Using the extraction procedures of Ogur and Rosen (23), the nucleic acid content was determined for leaf homogenates and for the series of pellets obtained by centrifuging at successively higher speeds. One such analysis is listed in table VII. The nuclear fraction was removed by sharkskin filter paper in this homogenate, so the figures refer primarily to cytoplasmic nucleic acid. In contrast to the difficulties encountered with extracts from whole chloroplasts, the extracts obtained from the smaller classes of particles and from the cytoplasm gave peaks at 260 mμ characteristic of nucleic acid.

The results show that about 40 % of the total

TABLE VI

COMPOSITION OF PURIFIED CHLOROPLASTS FROM TOBACCO LEAVES

ANALYSIS	PER MG CHLOROPLAST N	PER CHLOROPLAST
	MG	MG
Nitrogen	....	6×10^{-10}
Dry weight	10	6×10^{-9}
Chlorophyll	0.8 –1.0	$5–6 \times 10^{-10}$
Phosphorus	0.05	3×10^{-11}
Iron	0.005–0.015	$3–9 \times 10^{-12}$
Nucleic acid P ...	0.002–0.005	$1–3 \times 10^{-12}$
Nucleic acid N ...	0.004–0.01	$2–6 \times 10^{-12}$

TABLE VII

NUCLEIC ACID DISTRIBUTION IN A TOBACCO LEAF HOMOGENATE *

FRACTION	NUCLEIC ACID P/ML HOMOGENATE	DISTRIBUTION OF TOTAL NUCLEIC ACID	NUCLEIC ACID P/MG N	NUCLEIC ACID P/MG NON-PLASTID N **
	μG	%	μG	μG
Original homogenate	7.60	100	14.9	23
76×g sed.†	0.50	6.5	7.5	26
400×g sed.†	0.74	9.7	7.9	37
8,000×g sed.†	1.44	18.9	16.7	27
22,000×g sed.††	0.40	5.3	18.1	24
Total particulate	3.08	40.4	...	..
Supernatant	4.14	54.5	16.2	16
Recovery	7.22	94.9	...	..

* Homogenate prepared by blending, then straining through sharkskin filter paper. Nuclei removed by passage through sharkskin filter paper.
** Assuming no nucleic acid in the chloroplasts.
† 12 minute sedimentation.
†† 45 minute sedimentation.

nucleic acid in a homogenate freed of most of the nuclei is located in the particles, and close to half of this particulate nucleic acid is found in the particles that are rich in catalase and that sediment between 400 and 8,000×g. About 10% of the homogenate nucleic acid is found in the chloroplast-rich fraction, and only 5% in the very small particles. These figures on the distribution of nucleic acid are of course subject to the same errors, due to incompleteness of the separations, as was pointed out for catalase.

As might be expected from the analysis of purified whole chloroplasts, the fraction consisting mainly of chloroplasts is relatively poor in nucleic acid per mg N, compared to the next smaller particles which are rich in catalase and in nucleic acid. The smallest particles (22,000×g pellet) apparently have about the same concentration of nucleic acid as the catalase-rich particles.

DISCUSSION

In this study of the particulate organelles of leaf cells an attempt has been made to apply two criteria which are commonly used as evidence for the presence or absence of a particular enzyme activity or component from a given class of particles. The first is concentration or dilution of the activity, or other property under investigation, relative to its concentration in the whole homogenate (Schneider, 30). If the activity is concentrated relative to the homogenate level, then it is almost certainly localized in the fraction under consideration; if it is diluted, then it is probably absent from the fraction. The other criterion consists in the preparation of smaller amounts of highly purified particles, and measurement of any residual activity left in the pure preparation (Dounce, 9). Whereas previous work has indicated chloroplasts to contain catalase, cytochrome oxidase, and nucleic acid, the application of the above criteria in the present investigation strongly suggests that none, or only very small amounts, of these can be considered as present in the intact chloroplasts. On the other hand, the data do show by these same criteria that chlorophyll and the Hill reaction are present in the chloroplasts, and catalase and cytochrome oxidase are localized in the next smaller particles, probably mitochondria. Nucleic acid concentration is also high in these smaller particles.

In these two enzymes and nucleic acid, then, there is a measure of the degree of contamination of a chloroplast preparation by the smaller particles. Using these as markers, it is possible to see more clearly the difficulties found in separating chloroplasts from the smaller particles by centrifugation. By the time 90% of the plastids are down, 40% of the catalase and, inferentially, the small particles have also been sedimented under our conditions. Furthermore, if the chloroplasts were to be disrupted as by mortar and pestle grinding, then essentially no separation of the small particles from the chloroplast fragments could be accomplished by centrifuging. Only by using a grinding procedure that permits the majority of the plastids to remain intact, and selecting an appropriate centrifugal force, can separation of plastids from the smaller particles be achieved. Without such a separation, it is evident that activities and components would be ascribed to the chloroplasts which actually are localized in the smaller particles.

It seems likely that the particulate fraction rich in catalase contains mitochondria as a major component. It is the site of the respiratory enzyme, cytochrome oxidase, which is found in the mitochondria of animals (30), and etiolated plant tissues (17, 22). It contains catalase, as do animal mitochondria (30). The morphology of many of the particles is that which would be expected for mitochondria, and they are stained by Janus Green B at low concentrations. The sedimentation of the fraction is roughly that which would be expected from mitochondria in other tissues, although the particles do tend to come down at somewhat lower forces than has usually been observed. This might be

due, in part, to clumping caused by the M/15 phosphate buffer used in their preparation, together with the unknown electrolyte content of the vacuolar fluid of the leaf cells, which is liberated into the homogenate as the leaf is ground. We have not yet been able to show an active succinoxidase with preparations of these small particles; however, the absence of such an enzymatic activity could result from any number of as yet unfulfilled conditions in the isolation or measurement process.

The catalase-rich fraction is seen to have the highest concentration of nucleic acid of any of the particles examined. In animal tissues, this would be regarded as evidence that high salt had caused the submicroscopic particles to clump together so as to sediment together with the mitochondria (30). In view of the electrolyte content of the homogenates used here, this possibility cannot be eliminated. Alternatively, it is possible that leaf mitochondria and microsomes differ from those of animals in this fundamental respect. Further work with less acid leaves than tobacco, ground in entirely non-electrolytic media, is necessary before a decision can be made between these two alternatives. Such experiments might also result in cleaner separations of all three classes of particles: chloroplasts, mitochondria, and submicroscopic particles.

Our data on the RNA distribution in tobacco leaf homogenates corroborate those of McClendon (19) in showing a dilution of RNA where chloroplasts are concentrated. Comparisons of nucleic acids found in smaller particles in the two sets of preparations is difficult, since McClendon used different centrifugal forces to separate the fractions. In one experiment, McClendon's smallest centrifugal fraction (C_3, table II) showed a marked enrichment in RNA per mg protein N compared to the larger particles. However, in 6 experiments, our smallest particulate fraction (brought down at 22,000 × g for 45 minutes) contained from 16 to 40 γ RNA-P per mg non-plastid N, where the mitochondrial fraction (brought down at 10,000 × g) had from 15 to 36 γ RNA-P per mg non-plastid N. In each experiment, there was no marked difference between mitochondrial and microsomal nucleic acid content, in spite of the variability between experiments. It is clear that the smallest particle fraction failed to show an enriched content of RNA. These smallest particles are characterized as yet only by the combination of low catalase activity, slow sedimentation, and moderate nucleic acid content. Their function, or even origin within the cells, is still obscure.

Our data agree with those of McClendon (20) and Webster (35) as to the distribution of cytochrome oxidase in tobacco leaf homogenates. McClendon (20) was unable to find a similar marked enrichment or dilution of catalase activity per mg N in the centrifugal fractions of tobacco leaf homogenates which he investigated. This contrasts with our finding that up to an 8-fold enrichment of catalase occurs in the particle fraction just smaller than chloroplasts. In agreement with McClendon, we find that in homogenates prepared by blendor treatment the sedimentation of DNA parallels that of chlorophyll quite closely. With mortar and pestle ground homogenates, however, a clear centrifugal separation of the two is possible. We thus see no need to postulate, as McClendon has done, an "adsorption of DNA onto the chloroplasts" (19). We believe that the slower sedimentation of DNA in blendor homogenates can be correlated with the published microscopic observations of Weier and Stocking (37) which we have also made with our preparations: that is, that any blendor treatment, no matter how brief, causes nuclei to be elongated into long, thin, cohesive strands. It is obvious that a nucleus in the form of a thin strand will not sediment as rapidly as one with the same volume in the form of a sphere.

As shown in table I, we were able to eliminate nuclei from our preparations by passage through sharkskin filter paper. McClendon (19) was using homogenates routinely strained through sharkskin filter paper. It seems evident that nuclei under his conditions must have been able to pass through the filter paper, however. His homogenates (tables II and III, pp. 278 and 281) contained about 5 γ DNA-P per mg protein N, whereas ours at best, even when strained through cheesecloth and glass wool, had only 1 γ DNA-P per mg protein N. In view of the observed retention of DNA from our homogenates by filter paper, it may be that the nuclei were retained on the cellulose walls of the broken cells, even when the homogenates were strained through cheesecloth and glass wool. The differences between the two sets of preparations are obscure at the moment, and it is evident that further work is necessary to determine the conditions under which nuclei are or are not held back by the filtration techniques designed to eliminate unbroken cells.

The amount of nucleic acid found in the preparations of washed chloroplasts is quite small, and sets a fairly definite upper limit for the nucleic acid content of these plastids. The highest nucleic acid content found in one preparation was 5 γ P; but much more often the figure was 2 to 3 γ nucleic acid phosphorus/mg N. It is possible that our chloroplasts actually had no nucleic acid, since a small amount of nuclear contamination would be sufficient to give the values found. It would seem from the sedimentation curves presented that nuclei would be very difficult to remove when preparing chloroplasts from a blendor homogenate. Repeated passage of the homogenate through the sharkskin filter paper was used to remove nuclei. In addition, the nuclei can be seen to clump together, or to have extraneous matter clumped on their surfaces, after some centrifugation. These are then easier to remove by low speed centrifugations than the data on sedimentation from the original homogenate would indicate. In spite of this, it is not certain that all of the nuclei were completely removed from the preparations of purified chloroplasts. Microscopic examination always showed a very few structures which would stain with analine blue, and these may have been nuclei or emptied protoplasts. The

presence of DNA in the chloroplast preparations might seem to show that a large part of the nucleic acid found is indeed due to residual nuclei in the preparation. It is also possible, however, that DNA might be a real, though minor, constituent of isolated chloroplasts. If so, this would be consistent with the cytological observations of Metzner (21), who found DNA to be present in chloroplasts of mature leaves.

Chloroplasts, of course, are cytoplasmic bodies that can be observed to divide independently of the nucleus, and maternal inheritance of chloroplast characters shows them to be at least partially independent of nuclear control. The presence of appreciable amounts of nucleic acid has always been associated with self-reproducing entities in the past. The fact that preparations of purified chloroplasts have such low and possibly non-existent nucleic acid content is unusual from this point of view. The possibility cannot be eliminated that nucleic acid, originally contained inside the chloroplasts in the intact leaf, was removed by the homogenization and washing process. A number of observations indicate that large molecular weight substances are able to pass into and out of nuclei (1, 6, 33). However, only further experiments will be able to determine whether chloroplasts are similar to nuclei in being freely permeable to nucleic acids, enzymes, and proteins.

Assuming for the moment that the low nucleic acid content of our washed chloroplasts represents the true state of affairs inside the cell, it can be pointed out that chloroplasts in mature leaves have stopped dividing and enlarging (Strugger, 34). Chloroplasts and proplastids in much younger leaves are bodies that are capable of increase in size, and of division. It may be that, in the process of maturation, chloroplasts lose their nucleic acid content; or that the nucleic acid is stable and constant in amount and the increase in protein and pigments during plastid growth dilutes the amount of nucleic acid present to an insignificant proportion in comparison to the total N.

Summary

Sedimentation of chlorophyll and DNA from tobacco leaf homogenates has been studied as a function of the method of grinding the leaves. It is found that the majority of mature chloroplasts are left intact by brief blendor treatment, whereas about 50 % are disrupted by grinding the leaves with a mortar and pestle. Nuclei are left relatively intact by mortar and pestle grinding, but are strung out and disrupted by the blendor treatment. Curves are presented showing the sedimentation of chloroplasts and nuclei as a function of the centrifugal force employed.

From the chlorophyll/N ratios of successive pellets, it can be inferred that smaller particles which are not chloroplasts or fragments of chloroplasts occur in the homogenates. These smaller particles are rich in catalase activity, have a large amount of nucleic acid, and resemble mitochondria in appearance. Still smaller particles, on the border of visibility, can be centrifuged out of solution, and are found to be poor in catalase and have slightly less nucleic acid than the intermediate particles. The total amount of protein N found in these two kinds of smaller particles has been estimated, and compared with the total leaf protein found in chloroplasts, or in soluble form.

Conditions for separating chloroplasts from the smaller particles, and from nuclei, are established. These purified chloroplasts are then found to have little or no catalase activity, little or no cytochrome oxidase activity, but full Hill reaction activity compared to the original homogenate, some phosphorus and iron, and very little nucleic acid. The nucleic acid present according to the limited tests applied could be, either in whole or in part, DNA.

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THE ROLE OF VITAMINS AND MICRONUTRIENT ELEMENTS IN THE NUTRITION OF THE APICAL MERISTEM OF PEA ROOTS [1, 2]

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That cellular division and differentiation of young meristematic plant cells may be directly influenced and controlled by the presence of mature, differentiated, vascular tissues was reported many years ago by Haberlandt (11, 12). Support for the view that mature tissues are essential for the activity of meristematic tissues has come from the numerous reports (3, 7, 16) of failure to culture successfully excised root meristems lacking mature vascular tissues. It was early accepted in root culture work that a minimum tip length of several millimeters was essential for successful culture of transferred roots. Excised root tips which included only root cap, apical meristem, and a short zone of undifferentiated tissue proximal to the apical meristem usually would not survive in a medium which was apparently adequate for the growth of longer root tips which included mature vascular tissues. Chambers (7) reported the growth of squash root tips greater than 1.0 mm in length in a sterile nutrient medium, but found that tip fragments less than 1 mm in length, when cultured in an agar drop, separated into individual cells and formed no root.

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Scheitterer (19), using pea root tips grown in a sterile nutrient medium, found that the elongation of the excised tips was dependent upon the initial length of the excised root. She reported that root tips 0.5 to 1.0 mm in length grew up to 6 times the original length in 6 days in dilute Knop's solution containing 2 % glucose. Although she believed that the tip controlled differentiation of the primary tissues, Scheitterer could find no evidence to support Haberlandt's view that the mature tissues controlled cellular division in the meristem.

Gautheret (9) reported a 50 to 150 fold increase in initial length of 0.5 to 1.0 mm excised tips of corn roots cultured 3 months in a dilute Knop's solution containing 2 % glucose and cysteine hydrochloride.

Robbins, Bartley, and White (16) studied in some detail the influence of the size of the initial excised tip on its subsequent elongation in culture. With corn roots, they found that only roots 1.0 mm or longer could be predicted to grow normally in culture. Approximately 50 % of the tips 0.5 mm long grew, and shorter tips were even less likely to elongate.

Bonner and Addicott (3) in their study of the nutrition of excised pea roots in culture reported that tips 0.5 mm or less in length very rarely grew as normal roots. Tips 3 mm or longer grew as normal roots whose elongation rate was not affected by further increase in initial length. They indicated that "the development of vascular tissue must depend upon the presence in the tip of well defined prospective vascular tissue, since such tissue was not formed by tips containing only the initial zone."

Recently, Brown and Wightman (5, 30), in their analysis of meristematic activity based on total cell number in pea roots grown in nutrient culture, have found evidence that the mature differentiated tissues of the root control cellular division in the meristem by providing materials synthesized in the mature cells. They suggest that cellular division in the meristem is dependent upon two sets of metabolites, one set from the meristem itself and a second set provided from the mature tissues of the root. They have confirmed earlier observations (1, 3) that thiamin and nicotinic acid affect cellular division in the pea root meristem and have suggested, further, that thiamin may play a primary role in cell division in the meristem or an indirect role through an effect on the metabolism of mature tissues.

In only one case, reported by White (25), was there evidence to suggest that culture of the isolated meristem region is possible. White was successful in culturing excised tips of wheat roots as short as 0.1 mm in length in hanging drops of nutrient medium, carrying a few tips through several transfers.

In view of the manifest importance of the idea that mature, differentiated, vascular tissues influence cellular division and differentiation in the younger meristematic tissues, it was desirable to attempt to isolate and culture the embryonic tissue of the apical meristem of the pea root. The purpose of the present study was to determine the nutritional requirements of the isolated apical meristem itself and to establish the role of the mature, differentiated tissues in controlling cellular division in the meristem as well as the subsequent enlargement and differentiation of cells derived from it. A histological study of the differentiation of tissues derived from isolated half mm tips of pea roots will be reported elsewhere.

Materials and Methods

Excised roots of the garden pea, *Pisum sativum*, variety Alaska (Asgrow Strain No. 64010), were used in all experiments. The seeds were surface-sterilized in 0.1 % $HgCl_2$, rinsed in sterile water, and germinated in the dark in Petri dishes at 25°C for 48 hours. Five-mm tips were then excised, transferred to nutrient medium in 10-cm Petri dishes, 4 roots per dish, and grown in the dark at 25°C for 1 week. First-transfer primary root tips of various lengths were excised aseptically from these isolated roots, using a flame-sterilized, sharpened, steel needle under a dissecting microscope with 40× magnification and a suitably calibrated ocular micrometer. Transfers were then made with a flattened platinum wire. Relatively large numbers of extremely small root tips were successfully handled individually by transferring each excised tip to 1 ml of nutrient agar medium contained in a small modified Syracuse dish (U. S. Bureau of Plant Industry Model watch glass, 27 mm diameter). Eight such dishes fit snugly in a standard 10 cm Petri dish, and each root could be examined and measured accurately with the dissecting microscope during the course of the experiment, without contamination. About 100 roots were excised conveniently in a 2 hour period. For each treatment, usually 16 roots were used, with 6 treatments per experiment. Treatments were replicated at least 3 times in different experiments. Transfers were made in diffuse daylight with the transmitted beam of the microscope screened with a blue glass filter. Roots were returned to the dark at 25°C for the period of the experiment. Measurements made with the dissecting microscope at 40× were accurate to 50 μ.

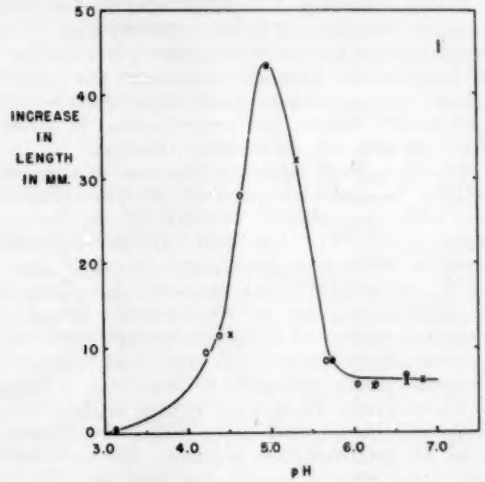
In initial experiments, the medium reported by Bonner and Devirian (4) (henceforth referred to as Bonner medium) as optimum for the growth of pea roots, modified from Bonner and Addicott (3), was used. The initial Bonner medium is shown in the second column of table IV. Difco Bacto-agar was added routinely to the medium at 0.5 %. In certain experiments noted below, specially purified agar (prepared by Difco Laboratories) was used. Pyrex redistilled water was utilized for all media and all reagents were of C.P. grade. Culture dishes were cleansed by soaking in dichromate cleaning solution, rinsed thoroughly with tap water, distilled water and redistilled water, and then air dried. Modification of the Bonner medium involved changes in concentration of individual components, replacement of components, adjustment of pH, and the addition of both inorganic and organic constituents. Such changes are described in detail below. Purification procedures used in the

study of the inorganic nutrition are also described at the appropriate point.

Experimental Results

Control of pH for Optimum Root Growth: Isolated roots grown in sterile nutrient culture tolerate a relatively narrow pH range for their optimum elongation (6, 26). Careful control of the pH of the medium therefore becomes an important aspect of any nutritional study with isolated roots in culture. A number of experiments were carried out initially to establish the optimum pH of the medium for elongation of isolated Alaska pea roots. Five-mm tips from isolated roots grown 1 week in culture were excised and transferred to 20 ml of liquid Bonner medium in 125-ml Erlenmeyer flasks. The pH of the medium was carefully adjusted, after the medium had been autoclaved and cooled, by the drop-wise addition of sterile 0.01 *N* HCl or 0.01 *N* KOH to the desired pH as determined electrometrically with the Beckman pH meter. It was found that adjustment after autoclaving was essential, as the pH of the medium may be markedly changed by the autoclaving procedure. In figure 1 are summarized the results from several separate experiments. The optimum pH for growth in the Bonner medium is approximately pH 5.0 or between 4.8 and 5.2. Any change in hydrogen ion concentration on either side of this optimum drastically reduces root elongation. The original Bonner medium, made as described by the authors, has a pH of about 4.8 after autoclaving, but has little buffering capacity and quickly changes pH upon the addition of small amounts of acid or base. It is essential that the initial pH of the medium be as close to pH 5.0 as possible in order to assure optimum root elongation. During root growth the pH of the medium tends to drop slowly, but the change is usually not sufficient to affect elongation markedly during a weekly passage. As the result of these tests with 5-mm roots, pH 5.0 was chosen for all subsequent studies, and adjustment of the medium to this pH was made aseptically following autoclaving and after all other additions or changes in the medium had been made.

Effect of Initial Root Tip Length on Elongation: The reported observation that successful culture of root tips depends upon the initial length of the tip was reexamined at the outset, in view of the important implication concerning the possible role of mature tissues in the nutrition of the meristem. First-transfer root tips of different lengths, from 0.5 to 10 mm including root cap, were excised from 1-week-old, isolated, pea roots in culture, transferred to Bonner medium in large Petri dishes, and grown in the dark. After 1 week, final lengths were measured and average root lengths determined. In figure 2 is represented graphically the relative increase in length in 1 week, i.e., final length divided by initial length, plotted against the initial length. Five-mm tips grew about 8 times their initial length in 1 week—to about 40 to 45 mm. Ten-mm tips (not shown in figure 2), the tip-length routinely used for weekly transfer of cultured roots, usually show about a 5 to 6 times increase over the initial length in a week. On the control Bonner medium, 2-mm tips appear to show the greatest increase in relation to initial length—i.e., about 9.5 times. This sizable increase over the initial



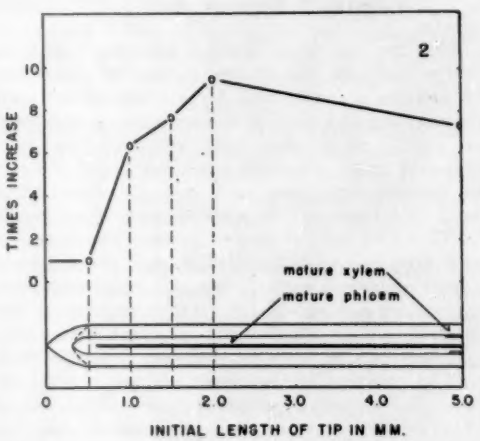


Fig. 1 (*left*). Increase in root length in one week in Bonner media of different initial pH. Initial length of excised pea roots was 5.0 mm. Each point is based on the average increase in mm of 20 roots.

Fig. 2 (*right*). Increase in length in one week (final length/initial length) of excised, first-transfer pea root tips of various initial lengths grown in Bonner medium in the dark. Note the lack of elongation of half-mm tips. The diagram below the curve represents a typical pea root in longitudinal view showing the position of mature xylem and phloem and of root cap and apical meristem in relation to the initial length of the root tips used in these experiments.

length reflects the relatively large proportion of the cells in the short tips which are still capable of elongation. In the longer root tips the number of unelongated cells is proportionately much smaller. In these experiments, the observation of Bonner and Addicott (3) was confirmed that 0.5 mm tips consistently fail to grow but become diffuse and disorganized masses of cells showing no organized development.

It is interesting to note from anatomical studies of these roots that 5-mm pea root tips usually include mature phloem and xylem tissue, as is shown in the root diagram drawn under the graph in figure 2. Shorter root tips, down to and including 1.0-mm tips, have no mature xylem present, but possess mature phloem tissue. Half-mm tips, however, possess no mature vascular tissues whatsoever; the tip includes only the root cap, with an average length of 300 μ, and about 200 μ of the essentially undifferentiated primary meristematic tissues including the apical initials and the subjacent procambial cone.

That half-mm tips would not grow on a medium which supports the growth of longer root tips might be due to one of several reasons: (1) the half-mm tip lacks certain metabolites necessary for cell division which are present in and provided by the mature tissues of the longer root tips; (2) in the half-mm tip, the proportion of damaged to undamaged tissues is so great that oxidations occurring at the damaged surface prevent normal cellular division in the meristem; (3) the rate of diffusion of essential metabolites from the tissues is so great as to drain the half-mm tip of materials needed for its cell division; or (4) the effective absorbing surfaces of the half-mm tip are too small to provide materials from the medium at a rate necessary to allow normal meristematic activity.

That the problem is chiefly a nutritional one soon became strikingly evident when it was discovered that the addition of autoclaved Difco yeast extract at a concentration of 1 gm/l of Bonner medium permitted the growth of half-mm tips into well organized, elongating roots. On such a medium about 80 % of the half-mm tips grew and increased their initial length 11.5 times in one week to an average length of 5.75 $\pm$ 0.92 mm. Vascular tissues differentiated in these roots and, when transferred, such tips appeared to grow as normal roots in culture. Apparently, certain nutrients essential for cellular division in the meristem, and usually available in longer excised root tips, were lacking from the half-mm tips and these could be provided by the simple addition of yeast extract. Subsequent experiments were carried out to determine the nature of these requirements and thus to establish a synthetic medium for optimum growth of the half-mm tips. In the preliminary experiments it became apparent that the nutritional factors of major importance include vitamin requirements, micronutrient element requirements, and optimum sucrose concentration.

For clarity of presentation of the results, it is necessary at this point to anticipate the fact, which became evident during the course of experimental work, that certain micronutrient elements are essential for pea root elongation and that conclusive demonstration of this fact necessitated special purification procedures. In all further experiments to be reported, therefore, the control medium was made from salts each purified separately of heavy metals by copper-hydrogen sulfide co-precipitation, following the procedure of Meagher (14). The procedure described is especially effective in the removal of traces of molybdenum (Mo), but also results in the removal of gross heavy metal contamination.

A 0.2 M stock solution of each of the five macronutrient salts used in the Bonner medium was made up, using redistilled water. One ml of 1 % $CuSO_4 \cdot 5 H_2O$ was added per liter of solution, which was then saturated with H_2S. After standing 12 hours, the precipitate was filtered off, additional copper sulfate solution was added, and the process repeated. Tests were made with H_2S on the final solution to assure complete precipitation of the copper. Excess H_2S was removed by aeration and boiling. This procedure effectively removed Mo and certain other heavy metals. Spectrophotometric determinations of Mo using the thiocyanate-stannous chloride test modified from Sandell (18) showed the Mo content of the complete medium minus Mo to be below the level of sensitivity of the test, i.e., less than 1 μg/l of solution. Dithizone tests modified from Stout and Arnon (21) showed total heavy metal concentration to be about 0.01 to 0.1 ppm. The elemental constitution of this heavy metal fraction was not determined by chemical tests, but the growth responses elicited in experiments to be described suggest that the level of zinc and manganese was low and copper relatively high. The control medium used in all experiments described below contained the following components: five macronutrient salts purified as above, ferric chloride (reagent grade), sucrose, and purified agar. It lacked added vitamins and micronutrient elements.

Vitamin Requirements: The two B vitamins known to be required by pea roots, thiamin and nicotinic acid, were already provided in the Bonner medium (table IV). Additional vitamins reportedly present in Difco yeast extract were tested by inclusion in the medium either separately or in groups. No significant response was elicited upon addition to the control medium of pyridoxine hydrochloride, biotin, riboflavin, indoleacetic acid, calcium pantothenate, or adenine, added separately or together in a range of concentrations which, from reports in the literature, might be expected to be within the effective range for optimum plant response. During subsequent experiments, it became clear that the levels of the two vitamins, thiamin and nicotinic acid, provided in the original Bonner medium and used in the control medium, were well below the concentrations required for maximum growth of the half-mm tips. In table I are presented data showing the clear response to the addition of optimum amounts of these vitamins. Optimum vitamin concentration is about

ten times that in the original Bonner medium. Although critical cell counts have not been made, it is apparent from histological studies that the response to the combined vitamin supplement is initiation of cellular divisions. In figure 3 is illustrated a typical series of roots, grown in 1-ml dishes for one week.

It is evident that unlike the 5 to 10 mm first-transfer, pea root tips, the half-mm tips have little or no stored supply of the vitamins, and cellular division in the meristem does not proceed without an external supply of both thiamin and nicotinic acid. Such dependence is strikingly demonstrated in these experiments by the all or none response to the addition of both vitamins. Either vitamin alone is ineffective in eliciting cell divisions. In subsequent experiments, it was found convenient to measure root tip length on the vitamin-less medium as the control for comparison with growth on other media.

Several attempts were made to determine whether the growth of half-mm tips was improved by any of a range of concentrations of pyridoxine hydrochloride, a vitamin specifically required by isolated roots of a number of plant species. No requirement for pyridoxine could be demonstrated (table I).

INORGANIC NUTRITION: In early work with the culture of isolated roots in nutrient media, the significant role of the trace metals in the nutrition of isolated plant organs received relatively little attention. Robbins, White, McClary and Bartley (17) emphasized the probable importance of trace amounts of mineral constituents in the nutrient medium for culturing tomato roots. White (27) found manganese, zinc, boron and iodine essential for growth of isolated tomato roots. Eltinge and Reed (8) described the effect of zinc deficiency on isolated tomato roots in synthetic culture solution. Glasstone (10), using special purification procedures, studied in some detail the inorganic micronutrient requirements of isolated tomato roots grown in culture, and concluded that iron and copper added to the medium in trace amounts were the only elements required at levels greater than 0.001 ppm. Recently, Boll and Street (2) have reported the stimulatory effect of molybdenum and copper on the growth of excised tomato roots when added in extremely low concentrations. As pointed out by White (29), many investigators in the field of tissue culture, proceeding on the established knowledge of micronutrient requirements for the growth of most higher plants, have included some or all of these known elements in the medium as a routine precaution. Little precise information is available at present concerning the specific elemental requirements of most isolated plant parts grown in culture.

TABLE I

RESPONSE OF HALF-MM PEA ROOT TIPS TO THE ADDITION OF YEAST EXTRACT AND TO ADDED VITAMINS. AVERAGE INITIAL LENGTH OF TIP: 525 $\mu \pm 11$

ADDITIONS TO CONTROL MEDIUM * (LACKING VITAMINS AND TRACE ELEMENTS)	% OF TOTAL WHICH GREW AS ORGANIZED ROOTS	AV. FINAL LENGTH IN μ AFTER 8 DAYS, WITH STANDARD ERROR	FINAL LENGTH/ INITIAL LENGTH
None	0	1585 ± 66	3.2
Yeast extract (Difco), 1 gm/l	80	5771 ± 922	11.5
Nicotinic acid **	81	2097 ± 87	4.2
Thiamin	70	1692 ± 113	3.4
Nicotinic acid, thiamin	80	4355 ± 233	8.7
Nicotinic acid, thiamin, and pyridoxine	80	3825 ± 146	7.7

* Control medium: five macronutrient salts especially purified, ferric chloride, 4 % sucrose, 0.5 % purified agar. (See improved medium, table IV.)

** Vitamin concentrations: nicotinic acid, 5 mg/l; thiamin hydrochloride, 1 mg/l; pyridoxine hydrochloride, 1 mg/l.

FIG. 3. Typical series of half-mm pea root tips grown for one week in the improved medium containing 4 % sucrose lacking micronutrient elements, showing the modified Syracuse dishes containing 1 ml each of nutrient medium. Average root length was 4450 μ.

The micronutrient requirements of excised roots grown in sterile synthetic nutrient medium have not been readily demonstrable even with careful purification procedures (2, 10), since mature tissues of transferred root tips carry over trace amounts from one passage to the next. Depletion of these trace amounts usually requires a number of transfers. It is not unreasonable to expect, however, that half-mm tips may contain extremely small amounts of the required micronutrient elements and will thus clearly manifest deficiencies after a single passage in medium lacking the required elements.

During the course of experiments concerning the nutrition of half-mm tips, it became evident that attention to the micronutrient requirements of pea root tips was of considerable importance if optimum root elongation was to be achieved. The original

Bonner medium included only iron as a specified addition, in the form of ferric tartrate at 1.5 mg/l. Other heavy metal requirements were presumed to have been met by contaminants of macronutrient salts.

In a study of the micronutrient requirements of pea roots, tests were first made using various sources of iron, including ferric sulfate, $Fe_2(SO_4)_3$, ferric chloride, $FeCl_3$, and ferric tartrate. Half-mm pea roots responded equally well to the inorganic forms of iron and, as the chloride is usually most likely to be free of contaminant trace metals, it was selected as the preferred form of iron for use in experiments concerning the micronutrient requirements. Reagent grade $FeCl_3$ was made up fresh for each new batch of medium and 1 ml amounts were added prior to autoclaving to give a final concentration of 1.5 mg/l. Subsequent tests made after a satisfactory medium had been worked out have indicated that the chloride is more effective than the tartrate as a source of iron for the elongation of half-mm tips.

The addition to the purified medium of trace amounts of all the other five known micronutrient requirements of higher plants—manganese, boron, zinc, copper, and molybdenum—produced a significant increase in root elongation, almost a 100% increase over that with vitamins alone. In table II are summarized the results of a number of experiments testing the effect of added trace elements in different combinations. Maximum elongation of half-mm roots was achieved using a medium with high levels of the vitamins in the presence of added micronutrient metals.

TABLE II

RESPONSE OF HALF-MM PEA ROOT TIPS TO THE ADDITION OF VITAMINS AND MICRONUTRIENT ELEMENTS *

ADDITIONS TO CONTROL MEDIUM **	AV. FINAL LENGTH IN μ AFTER 8 DAYS, WITH STANDARD ERROR	FINAL LENGTH/ INITIAL LENGTH
None	1358 ± 92	2.7
Nicotinic acid 5 mg/l and thiamin·HCl 1 mg/l	3875 ± 284	7.8
Vitamins plus:		
$ZnSO_4$, 1.5 mg/l	3832 ± 465	7.7
$MnSO_4$, 4.5 mg/l	4347 ± 363	8.7
$Na_2MoO_4 \cdot 2\,H_2O$, 0.25 mg/l	4047 ± 275	8.1
H_3BO_3, 1.5 mg/l	4365 ± 320	8.7
$CuSO_4 \cdot 5\,H_2O$, 0.04 mg/l	.....	..
Vitamins plus trace elements except:		
Zn	4950 ± 345	9.9
Mn	5000 ± 518	10.0
Mo	4225 ± 257	8.5
B	6000 ± 680	12.0
Cu	6800 ± 727	13.6
Vitamins plus trace elements, less $FeCl_3$	3158 ± 158	6.3
Vitamins and all trace elements	7400 ± 747 7108 ± 519	14.8 14.2

* Average initial length of tips: 525 μ. Averages based on 12 to 16 roots per treatment.

** Control medium: five macronutrient salts especially purified, ferric chloride, 4% sucrose, 0.5% purified agar. (See improved medium, table IV.)

The concentrations of micronutrient elements reported in table II were based on concentrations in White's medium (28) and the work of Boll and Street (2). Although measurement of the actual concentrations of each of the micronutrient elements in the final medium was not made, it is probable that the optimum concentrations reported in table II are within the most effective concentration range. Minimum concentrations for effectiveness were not determined, although in certain experiments in which the micronutrient element concentration was reduced, there was a reduction in final root length. Experiments in which each element was added alone (table II) indicate that no one trace metal was especially deficient, as no single added element increased root elongation over that achieved with vitamins alone. The separate omission of each of the three elements, zinc, manganese, and molybdenum, from the otherwise complete medium resulted in significant reduction of root elongation in each case. Omission of copper or boron separately showed no significant effect. The concentrations of the last two elements were not subject to control, however, as boron contamination from the Pyrex dishes probably occurred, and copper was utilized in the purification procedure itself. The omission of molybdenum, manganese or zinc resulted in a degree of elongation not significantly different from that in vitamins alone. The zinc requirement is a particularly interesting one, especially in the light of its known importance in auxin synthesis (20, 24). A detailed analysis of specific micronutrient requirements of pea root tips, will require further special purification of the salts and has not yet been carried out. It is clear, however, that the elongation of the tips is nearly doubled upon the addition of the five micronutrient elements to the vitamin-containing medium.

In the present study of the nutrition of the half-mm tips of pea roots, no attempt was made to modify the macronutrient constituents of the original Bonner medium. It is conceivable that modification in the direction of increasing the buffer capacity of the medium, by increasing the concentration, for example, of the phosphate, might increase the overall growth of roots through many passages. As this work is concerned with the optimum elongation of small tips over a relatively short duration, the effect of enhanced buffer capacity of the medium has not been analyzed.

SUCROSE CONCENTRATION: The concentration of sucrose in the medium has been shown by Street and McGregor (23) to influence markedly the growth of excised tomato roots in culture. In their study of the nutrition of 10-mm pea root tips, Naylor and Rappaport (15) reported that the sucrose concentration required for maximum root elongation varied from 4% to 8%, dependent upon the variety of pea. In view of these reports, experiments were run in which half-mm pea root tips were grown in the improved medium, containing the increased levels of vitamins, all trace elements at optimum concentrations, and iron

TABLE III

RESPONSE OF HALF-MM PEA ROOT TIPS TO VARIOUS CONCENTRATIONS OF SUCROSE *

SUCROSE %	AV. FINAL LENGTH IN μ AFTER 8 DAYS, WITH STANDARD ERROR	FINAL LENGTH/ INITIAL LENGTH
1	5775 ± 248	11.1
2	6075 ± 575	12.1
3	6850 ± 660	13.7
4	7475 ± 625	14.9
6	9150 ± 817	18.3
8	8125 ± 1193	16.3

* Improved medium as shown in table IV used with varying levels of sucrose. Averages based on results of two separate experiments.

as the chloride, but in which various levels of sucrose were provided. In table III are shown averaged results of two such experiments. The concentration of sucrose for maximum elongation of half-mm pea roots was found to be 6 %. Change of sucrose concentration on either side of this optimum resulted in slight but significant reduction in root elongation.

DISCUSSION

The improved medium developed from these experiments which will produce excellent growth of half-mm root tips of Alaska peas is given in table IV.

Successful culture of small fragments of the root tip, including little more tissue than the apical meristem and its immediate immature derivatives, makes untenable the earlier idea that differentiated tissues, especially vascular tissues, are essential per se for normal development of the apical meristem of the root.

TABLE IV

COMPARISON OF ORIGINAL BONNER-DEVIRIAN MEDIUM, INEFFECTIVE FOR GROWTH OF HALF-MM PEA ROOTS, AND THE IMPROVED EFFECTIVE MEDIUM *

COMPONENT	BONNER-DEVIRIAN (4) MEDIUM	IMPROVED MEDIUM
	per liter	*per liter*
$Ca(NO_3)_2 \cdot 4\,H_2O$	242 mg	242 mg
$MgSO_4 \cdot 7\,H_2O$	42 mg	42 mg
KNO_3	85 mg	85 mg
KCl	61 mg	61 mg
KH_2PO_4	20 mg	20 mg
Ferric tartrate	1.5 mg	...
$FeCl_3$	...	1.5 mg
Thiamin · HCl	0.1 mg	1.0 mg
Nicotinic acid	0.5 mg	5.0 mg
Sucrose	40 gm	60 gm
$MnSO_4 \cdot H_2O$	...	4.5 mg
$ZnSO_4 \cdot 7\,H_2O$	...	1.5 mg
H_3BO_3	...	1.5 mg
$CuSO_4 \cdot 5\,H_2O$	...	0.04 mg
$Na_2MoO_4 \cdot 2\,H_2O$	...	0.25 mg
Difco purified agar ..	...	5.0 gm

* Final concentrations of components given in weight per liter. Media autoclaved at 15 lb/inch² for 20 minutes.

The major changes in the medium making growth possible are four: 1) replacement of ferric tartrate by ferric chloride as a source of iron; 2) a tenfold increase in the concentration of both required vitamins—thiamin and nicotinic acid; 3) addition in trace amounts of five micronutrient elements: Zn, Mn, Mo, B, Cu; 4) an increase in the sucrose concentration from 4 % to 6 %. It seems, then, that half-mm root tips grown under the conditions of these experiments lack completely the two required vitamins and more than one of the trace elements. Presumably, 5-mm tips have adequate supplies of these requirements, owing either to synthesis or to residual storage. Residual storage of vitamins in large root segments is readily demonstrated (13). Half-mm tips show their dependence upon the synthetic medium supplied them

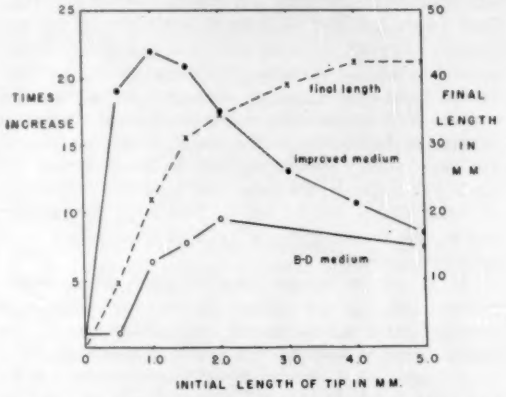


FIG. 4. Increase in length in one week (final length/initial length) of excised, first-transfer pea root tips of various initial lengths grown in the dark in the improved medium but with 4 % sucrose. The final lengths in mm are shown by the broken line. The curve from figure 2 is replotted and represents comparable root elongation on the original Bonner medium.

much more rapidly than do larger tips which do not lack stored materials.

From this point of view, it was thought interesting to repeat the earlier experiment concerning the importance of the initial length of the tip, using now the improved medium with 4 % sucrose. The results of such an experiment are presented graphically in figure 4, in which the relative increase in length (final length/initial length) is plotted against the initial root length. Final length in mm is also plotted on the ordinate to the right. The curve from figure 2 of relative increase in growth on the original Bonner medium is replotted in figure 4 for purposes of comparison.

Several interesting facts are noted from such a comparison. In the first place, tips 5 mm in length or longer grow about equally well on the two media. As progressively shorter tips are used, the constitution of the medium becomes progressively more important, and a striking difference in response to the two media is evident. In the improved medium, the elongation

of 1-mm tips in terms of relative increase is over three times that in the original Bonner medium. In tips 5 mm or greater, the older mature tissues of the root provide all the vitamins and trace elements needed for the growth of the tip for at least a week, and no difference in response is noted. Progressively shorter root tips are more dependent on the external supply of these requirements. In these experiments, 1-mm tips appear to be most sensitive to modification of the medium, and one is led to conclude that studies concerned with the nutrition of isolated roots would prove most profitable, and in fact, much simplified, if short root tips rather than long tips were utilized.

From figure 4 it is apparent that half-mm tips are a less desirable length for nutritional studies with respect to their growth response than are 1-mm tips. Although certainly more deficient in reserve materials than 1-mm tips and thus more dependent on the externally supplied medium, half-mm tips are probably limited in cellular division and elongation by the relatively high proportion of damaged to undamaged tissue. With respect also to the mechanical difficulties of securing half-mm tips, the longer, 1-mm tips, which are more readily excised, appear to be preferable for studies concerned with pea root nutrition. Survival of 1-mm tips is usually 100 %, indicating that at this length, the wound effect of excision is no longer seriously detrimental.

It is interesting to consider now the question whether the mature tissues of the root contribute special factors which control cellular division of the apical meristem and the subsequent processes of cellular differentiation. From the evidence presented here, one must conclude that the older tissues proximal to the apical meristem of the root serve as an immediate source of essential vitamins and trace elements, although these may originate elsewhere in the plant. The two added vitamins appear to be concerned directly in cellular division in the meristem. With respect to the micronutrient elements, the present experiments do not clearly distinguish between a direct role in cellular division or a secondary effect upon the subsequent elongation of the cells. It is not impossible that certain organic compounds, whose syntheses are dependent upon catalytic amounts of one or more of the trace elements, may be provided to the apical region from the older regions of the root where such syntheses actually occur. Evidence for such a view, which is implied by Brown and Wightman (5, 30), must await further detailed analysis of the role of the micronutrient elements. It is evident, however, that the apical region itself *can* carry on the synthetic processes necessary to produce new cells if the region itself is provided with the essential requisites of carbohydrate, vitamins, and elements. Supplying the tip with additional factors, at present unknown, might produce a still more rapid rate of root elongation than that achieved here, a possibility suggested by the work of Naylor and Rappaport (15). It is of considerable interest in this connection that the differentiation of tissues in the roots derived from half-mm tips does *not* always result in the typical, triarch, vascular tissue pattern observed in the larger pea root tips. In a number of cases studied, typical, symmetrical, diarch and even monarch vascular tissue arrangements have been formed. Whether the change in pattern is the result of isolation of the apical meristem from the inductive influence of biochemical products of the mature tissues or whether it is due to some other cause remains to be ascertained.

The idea of Haberlandt (11, 12) and many others that cellular divisions of the apical meristem and other meristematic tissues are dependent upon the provision by the mature, differentiated tissues, including, especially, vascular tissues, of a special "Teilungshormone" or "Leptohormone" can be reinterpreted in the light of these experiments. The apical meristem of the pea root is, in fact, usually dependent upon mature tissue for an adequate supply of thiamin and nicotinic acid, which the meristematic cells of the tip cannot synthesize. In this sense, these two vitamins coming from mature tissues behave as "Teilungshormones." That half-mm tips which lack mature phloem are completely deficient in vitamins suggests that these vitamins move to the region of the meristem in the mature phloem and must then move cell by cell in the undifferentiated tissues distal to the mature phloem tissues.

In a similar fashion, the trace elements must function in synthetic activities leading to normal cellular division and cell enlargement. When all the requirements of the meristem are provided directly, the mature, differentiated tissues of the root and the shoot may be dispensed with as a source of supply. The differentiated, mature, primary tissues of the root appear to possess no peculiar, unknown capacity for syntheses essential to the activity of the meristem which cannot be replaced from an externally supplied, synthetic, nutrient medium.

Summary

The nutritional requirements of the apical meristem of the pea root were determined directly by the development of a synthetic nutrient medium capable of supporting growth of excised half millimeter root tips, including root cap and approximately 200 μ of the essentially undifferentiated, primary meristematic tissues of the apical region, completely lacking mature vascular tissues. Modification of the pea root medium of Bonner and Devirian (4) included replacement of ferric tartrate with ferric chloride as an iron source, ten times increase in concentration of the required vitamins—thiamin and nicotinic acid, increase of sucrose concentration from 4 % to 6 %, and the addition of salts of the five micronutrient elements, zinc, manganese, molybdenum, boron, and copper.

In the absence of either or both vitamins, no root growth occurred. Addition of all five micronutrient elements to the vitamin-containing medium, otherwise purified of heavy metals, resulted in a 100 % increase in root length. The omission from the complete medium of any one of the three micronutrient ele-

ments, zinc, manganese, or molybdenum, resulted in a degree of root elongation similar to that in the medium containing only vitamins. Judging from the extreme sensitivity of half millimeter root tips to these omissions in the nutrient medium, it is concluded that such tips are practically devoid of the two required vitamins as well as of one or more of the essential micronutrient elements. It is pointed out that such extremely small tips are especially valuable in the analysis of nutritional requisites since they are depleted so readily of their externally supplied nutrient requirements. The mature, differentiated tissues of the root can be thought of as providing essential nutritional requirements which regulate cellular division in the meristem. Upon isolation of the meristem from mature tissues, such requirements must be provided by the external nutrient medium itself. All these requirements have been met in the improved medium reported here. In such a medium, the apical meristem itself can carry out all the synthetic processes necessary for the formation of new cells and their differentiation.

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ISOLATION OF CARBON-14-LABELED SEDOHEPTULOSE AND OTHER PRODUCTS FROM SEDUM SPECTABILE[1,2]

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The accumulation of sedoheptulose during photosynthesis has been reported for many of the succulent plants (1, 2, 3) and the widespread occurrence of this sugar (4, 5, 6) indicates a participation in the normal metabolic pattern of the plant. The experimental demonstration of its metabolic role in plants has been presented by Benson et al (7) and Axelrod et al (8). It is the purpose of this paper to present a procedure for the biosynthesis and isolation of sedoheptulose-C^{14}. During the course of this work, other C^{14}-labeled substances were also recovered, providing additional information on the metabolic activities of the *Sedum spectabile* plant.

The usual procedure for obtaining the sugar resulted in its isolation as the anhydride, sedoheptulosan (9). But, since sedoheptulosan is not an equivalent substitute in the plant for sedoheptulose (16), this procedure was followed only up to the conversion of the sugar to the sedoheptulosan, and then the free sugar was isolated by thick-paper chromatography.

EXPERIMENTAL

Sedum spectabile Bor. plants, which have a high sedoheptulose content (4), were exposed to $C^{14}O_2$ in a photosynthesis chamber (11) under the conditions given in figure 1. These plants, which had been grown in the greenhouse for about two months, were cut off at the base at 10:00 A.M. on a sunny morning and placed in water for the beginning of the biosynthesis. They remained turgid and apparently healthy during the entire labeling period.

Isolation of the sedoheptulose was carried out by the procedure presented in figure 1, the preliminary steps of which are similar to those used for the preparation of sedoheptulosan (9). Each step in the isolation process was followed by paper chromatography. The chromatography procedure employed at this point was similar to that of Benson et al (12), and the solvents were phenol-water in the first direction and butanol-propionic acid-water in the second direction. The sugars were identified by chromatography and by various spray tests described by Block et al (13). Sedoheptulose gave the characteristic blue color with orcinol-TCA spray upon heating the chromatogram at 100°C for at least 5 minutes (14). Acids were identified by R_f values and by rechromatography in ether-acetic acid-water (15). In the latter case there was coincidence of radioactivity with added carrier acids which were detected by bromocresol green indicator spray test.

Final separation of the free sedoheptulose-C^{14} was accomplished in milligram amounts by using thick paper chromatograms of Whatman No. 3 MM, 18½ × 22¾ inches. One dimensional development of a band of about 100 mg of the sugar mixture was used (16). Solvents flow so rapidly through this thick paper that good separation of closely related compounds such as glucose, fructose, and sedoheptulose cannot be accomplished. To overcome this, a 2½ inch strip of Whatman No. 1 paper was sewed along one side of the thick paper to act as a wick with only the thin paper dipped into the solvent (17). The solution of compounds to be separated was placed on a line on the thick paper very close to the sewed-on wick. In this system about 48 hours were required to develop a descending chromatogram; excellent separation of the sugars was obtained.

RESULTS AND DISCUSSION

The chromatogram of the original extract (fig 2) was similar to the long-term photosynthesis chromatograms of Benson et al (12). The main sugar products labeled with C^{14} were sedoheptulose, fructose, glucose, and sucrose. C^{14} activity in the sugar phosphates was present in a very small amount as compared to the free sugars. Citric, isocitric, malic, and glyceric acids were present in major amounts.

The chromatogram of the supernatant, after clarifying the original extract with activated charcoal followed by methanol precipitation, demonstrated that large amounts of sugars and some acids, primarily glyceric and citric, were left in the filtrate. The methanol-insoluble material was extracted with water to recover the organophosphate esters and, as shown by paper chromatography, also considerable amounts of the malic, citric, and isocitric acids as well as small amounts of some amino acids. A significant amount of labeled sugars was also present in the washings from the methanol precipitation.

ISOLATION OF SEDOHEPTULOSE-C^{14}: The established procedure for the final isolation of sedoheptulose, after concentrating the methanol extract, involves acid treatment for conversion of the sedoheptulose to sedoheptulosan (9). Although this form of sedoheptulose can be crystallized from a mixture of sugars, the sedoheptulosan has several disadvantages for biological work. By paper chromatography it was found that the first crystallization was often contaminated with glucose and repeated recrystallizations were necessary to obtain pure sedoheptulosan. This is difficult without added carrier when only small amounts of C^{14}-labeled material are available. But more important, the sedoheptulosan is a nonphysiologically active form of the sugar (10). Its conversion back to the free sugar is possible by acid treatment, but only in 20 % yield (9). The other 80 %

[1] Received November 18, 1953.
[2] Work performed under Contract No. W7405-Eng-26 for the Atomic Energy Commission.

remains in the solution as sedoheptulosan with the sedoheptulose, giving rise to the possibility of competitive inhibition of enzyme systems to be studied.

Thick-paper chromatography was utilized to separate the free sugar, sedoheptulose-C^{14}, from the sugar syrup after removing the methanol. After development in butanol-propionic acid-water the sedoheptulose portion of the chromatogram was carefully cut out, free of fructose and sucrose but still contaminated with some glucose. It was freed from glucose by first developing a radioautogram of the small strip containing the sugar. The sedoheptulose was eluted from the paper and rechromatographed in phenol-water to effect its separation from the remaining glucose (fig 3).

This sedoheptulose, when rechromatographed by two-dimensional technique, consisted of only one radioactive spot which gave a blue orcinol-TCA color test and which had R_f values of 0.44 in phenol-water and 0.22 in butanol-propionic acid-water. A convenient dye to use on the chromatogram with these solvents is croceine scarlet which has almost the same R_f values as sedoheptulose. Portions of sedoheptulose were eluted from the paper chromatograms as needed. The free sugar had a specific activity of 3.7 μc/mg as determined by the orcinol test for the quantity of the sugar, and by wet combustion to carbon dioxide and counting of the $BaC^{14}O_3$ (18). Degradation of the sugar indicated that, within the limits of the methods available, the activity was uniformly distributed. Ceric oxidation gave 13 % of the activity in carbon-2; periodate oxidation of the anhydride gave 15 % in carbon-4; periodate oxidation of the osazone gave 35 % in carbons 1, 2, and 3; 34 % in carbons 4, 5, and 6; and 12 % in carbon 7. Periodate

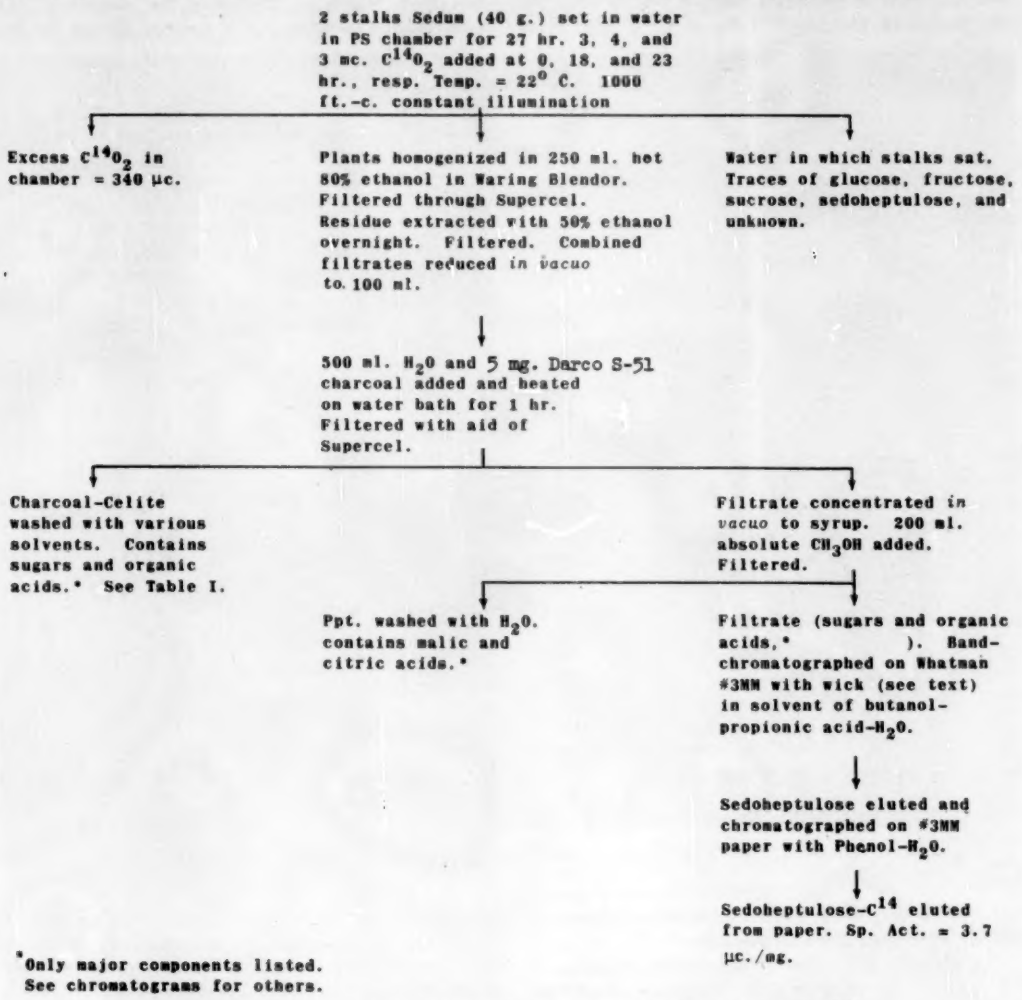
2 stalks Sedum (40 g.) set in water in PS chamber for 27 hr. 3, 4, and 3 mc. $C^{14}O_2$ added at 0, 18, and 23 hr., resp. Temp. = 22° C. 1000 ft.-c. constant illumination
Excess $C^{14}O_2$ in chamber = 340 µc.
Plants homogenized in 250 ml. hot 80% ethanol in Waring Blendor. Filtered through Supercel. Residue extracted with 50% ethanol overnight. Filtered. Combined filtrates reduced in vacuo to 100 ml.
Water in which stalks sat. Traces of glucose, fructose, sucrose, sedoheptulose, and unknown.
500 ml. H_2O and 5 mg. Darco S-51 charcoal added and heated on water bath for 1 hr. Filtered with aid of Supercel.
Charcoal-Celite washed with various solvents. Contains sugars and organic acids.* See Table I.
Filtrate concentrated in vacuo to syrup. 200 ml. absolute CH_3OH added. Filtered.
Ppt. washed with H_2O. contains malic and citric acids.*
Filtrate (sugars and organic acids,*). Band-chromatographed on Whatman #3MM with wick (see text) in solvent of butanol-propionic acid-H_2O.
Sedoheptulose eluted and chromatographed on #3MM paper with Phenol-H_2O.
Sedoheptulose-C^{14} eluted from paper. Sp. Act. = 3.7 µc./mg.
*Only major components listed. See chromatograms for others.

FIG. 1. Schematic outline of biosynthesis and isolation of sedoheptulose-C^{14}.

oxidation of the sedoheptitol gave 28 % in carbons 1 plus 7, and 69 % in carbons 2 through 6 (19).[3]

A specific figure for total yield of sedoheptulose has not been determined, since the isolation of sedoheptulose from all of the fractions has not been run through to completion. On the basis of the distribution of C^{14} in the original plant extract and of a comparison of the amounts of sedoheptulose isolated on 15 paper chromatograms, it was possible to estimate that the yield of sedoheptulose-C^{14} was at least 300 mg, containing more than 1 mc of C^{14}. Also about

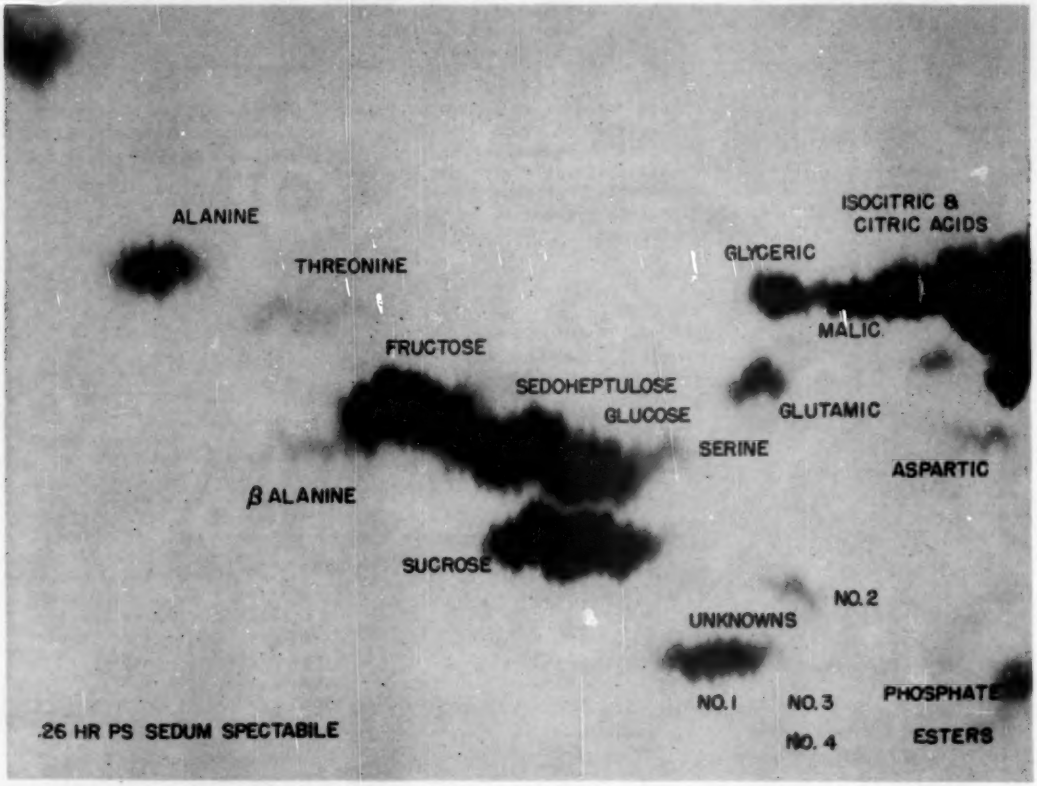

FIG. 2. Chromatogram of the original concentrate from the combined 80 % and 50 % alcohol extracts of minced *Sedum spectabile* leaves.

equal yields of glucose and fructose were obtained as well as more than 1 mc of sucrose.

An attempt was made to separate the mixture of free sugars, as their borate complexes, from the methanol extract by ion-exchange chromatography (20) using Dowex-1 in the borate form with potassium tetraborate solutions as eluting agents. Although it was found that sucrose and glucose could be quantitatively removed, it was not possible to separate the sedoheptulose and fructose under these conditions. That the two keto sugars come off the column as a single peak was indicated by paper chromatography of the constituents of each peak after removal of the borate. Since an exhaustive study of this problem was not made, it is possible that other ion-exchange chromatographic conditions may be found to effect a separation of these keto sugars.

C^{14} ACTIVITY RETAINED ON THE ACTIVATED CHARCOAL: In trial runs without C^{14}, activated charcoal was found very desirable for obtaining a clean solution from which to crystallize the anhydride. On making an approximate C^{14} balance during the bio-synthesis it was noted that a great deal of the activity had been retained in the charcoal. Furthermore, by studying the chromatograms after each step of the isolation procedure, it was noted that unknown number 1 was completely absorbed by the charcoal. In order to recover some of this activity the charcoal was washed in a column with continuous flow and the eluates were chromatographed (table I). By water washing, about 10 % of the total activity fixed by the plant, representing 1 mc of C^{14}, was eluted from the charcoal before the activity in the eluate approached zero. An aliquot of the water washing of the charcoal was analyzed by two dimensional paper chromatography similar to that used for studying the original

[3] Degradation was done at the Radiation Laboratory, University of California.

plant extract. The washings contained a large amount of sedoheptulose-C^{14}, and thus a substantial increase in yield of this sugar could be effected. Considerable malic acid was also recovered. The charcoal was further washed with 70 % ethanol, by which it was possible to elute about an additional millicurie of C^{14} activity consisting mainly of glucose, fructose, sucrose, and malic acid but having only a trace of sedoheptulose. A solution of 80 % phenol and 20 % water by volume eluted still another mc of C^{14} activity. The two major products in this eluate were malic acid and glyceric acid. No activity was eluted with ether or acetone.

If biosynthesis of sedoheptulose were the main objective, this could be accomplished by combining the original charcoal extract and the eluate from exhaustive water washing of the charcoal. The use of other charcoal has not been studied extensively, but a nonactivated bone charcoal was not satisfactory since it did not clarify the plant extract.

Unknown Compounds from Sedum Spectabile: The unknowns shown in figure 2 would appear to be polysaccharides because of their low R_f values. U_1 (unknown number 1) was lost by absorption on the charcoal. Chromatograms of substances eluted from the charcoal by alcohol showed a double spot at the location of U_1. The U_1 from the original plant extract, when heated with Dowex-50 in the acid form at 100°C for 1 hour, yielded, besides some original unknown, only one radioactive spot, which was identified as fructose. Thus it appears possible that U_1 may be a short-chain fructosan similar to inulin. U_2 appeared to be a very complex compound. It was not entirely absorbed by the activated charcoal, and that which was absorbed could best be eluted from the charcoal by phenol-water. No new labeled compounds were produced from it by treatment with a commercial phosphatase, Polidase S. Treatment with Dowex-50 in the acid form at 100°C for 1 hour caused some decomposition to a series of unknowns, having widely divergent R_f values.

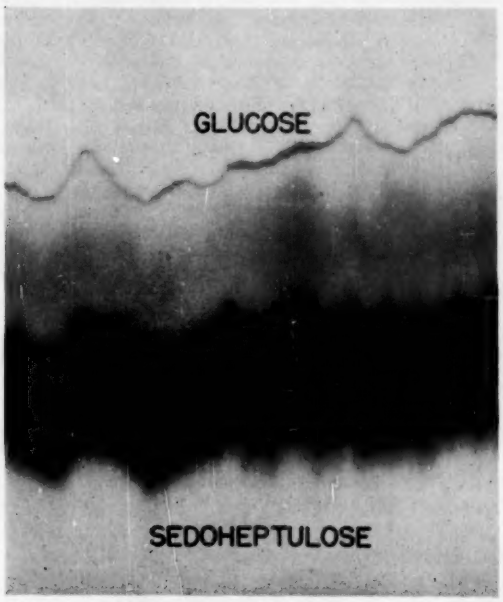

Fig. 3. One-dimensional chromatogram of the final separation of sedoheptulose from small amounts of glucose. This is a descending chromatogram of Whatman No. 3 MM paper with a Whatman No. 1 wick. The solvent front is off the paper at the bottom. The sharp line at the top of the figure is due to chemical fogging of the no-screen X-ray film by impurities in the phenol and it is not radioactive. Glucose lies between this line and the main sedoheptulose band.

Table I

Extraction of Carbon-14 Activity from Charcoal; Percentage of Total Activity *

Products	Eluate		
	Water	70 % alcohol	80 % phenol 20 % water
Sedoheptulose ..	32.7	trace	0.9
Glucose	14.9	17.8	2.7
Fructose	21.6	18.4	1.9
Sucrose	11.8	41.2	5.9
Malic acid	17.4	9.7	54.4
Glyceric acid ...	0.0	0.0	21.0
Phosphate area	0.8	0.5	4.1
Unknown 1	0.0	4.7 **	1.2
Unknown 2	0.3	0.0	3.3

* Each extraction procedure eluted approximately 1 mc of C^{14}. An aliquot of the extract was analyzed by the two dimensional paper chromatography procedure. Percentage of activity in each compound was on the basis of total C^{14}-activity on a chromatogram.

** Two spots.

Translocation of Sugars in Sedum Spectabile: The water which had held the *Sedum spectabile* stalks during the exposure to $C^{14}O_2$ contained a small amount of C^{14}-nonvolatile material after being acidified. Chromatography of this solution showed that small amounts of glucose, fructose, sedoheptulose, sucrose, and C^{14} activity which did not move from the origin of the chromatogram, had been translocated down the plant stalk into the water. Very slight traces of several other compounds were also present. The results show the downward translocation of sugars in the plant and indicate that sedoheptulose in the *Bryophyllum* species may also be transported downward, for metabolism in the roots. Such a translocation may be similar to that already demonstrated for sucrose (21).

Summary

1. Sedoheptulose-C^{14}, with a specific activity of 3.7 μc/mg and approximately uniform labeling, has been biosynthesized from *Sedum spectabile* and isolated, as the free sugar in milligram amounts, by thick-paper chromatography.

2. Fructose, glucose, sucrose, malic acid, and citric

acids have also been isolated by the procedure. An unknown was tentatively identified as a fructosan.

3. Sedoheptulose was translocated down the plant stalk, as were glucose, fructose, and sucrose.

The authors wish to acknowledge the cooperation of Dr. G. R. Noggle during the trial experiments for C^{14}-labeling of the plant material and the assistance of Mrs. Betty Ann Gwin throughout the work. We wish to thank Mrs. Lorel L. Kay of the Radiation Laboratory at the University of California for degrading the sedoheptulose-C^{14}.

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THE EFFECT OF AUXIN ON THE FLOWERING BEHAVIOUR OF WINTEX BARLEY AND PETKUS RYE[1]

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Investigations of the problem of auxin in relation to flowering have so far been confined to experiments in which solutions of growth substances have been applied to plants grown under favourable or unfavourable photoperiods.

Auxin has been shown to inhibit flowering in a number of short-day plants: Dostal and Hosek (4) with Circaea; Bonner and Thurlow (1) with Xanthium; Harder and Van Senden (7) with Kalanchoë; Leopold and Thimann (8) with teosinte; and also in the long-day plant Calendula by von Denffer and Grundler (12).

It appears that the only clear-cut instance of flower promotion by applied auxin is in the pineapple

[1] Received December 30, 1953.

(3, 13, 14). Small quantities of α-naphthaleneacetic acid or other active substances will induce inflorescence formation in plants that otherwise remain vegetative for several seasons. From the preliminary data of van Overbeek (14) it appears that the pineapple is a short-day plant.

Leopold and Thimann (8) describe the effects of α-naphthaleneacetic acid on the flowering behaviour of barley, variety Wintex, a long-day plant (2). Solutions of this auxin in low concentration in the order of 0.01 mg/l, when applied through cut leaves, increased the number of flower primordia on the developing ear over that of the controls. When the concentration was higher than 10 mg/l the number of flower primordia was decreased. These effects were accompanied by parallel changes in plant weight and axis length. Leopold and Thimann interpret these results as a " promotion " and " inhibition " of " flower initiation " respectively, taking the " relative abundance of flower primordia as a measure of flower initiation." From this and other observations they develop the hypothesis that in barley there is an optimal level of auxin for flowering, and that factors influencing the auxin level in the plant, will, under certain conditions, modify its flowering behaviour.

The experiments described in this paper were undertaken in order to confirm the effects of applied auxin on barley and to investigate the possibility of similar effects on rye.

Materials and Methods

Experiments with Petkus Rye: In 1950 a large scale experiment was carried out with rye, variety Petkus. The details of the experimental procedure followed that described by Leopold and Thimann for barley. Plants injected with auxin solution were treated as follows: the distal half of the blade of the youngest mature leaf on each plant was cut off with a razor and the remaining part of the lamina inserted in a small test tube containing one ml of auxin solution. The control plants were treated with water. A cotton wool plug was used to maintain the cut leaf in position and to reduce evaporation. The tubes were removed from the plants after five days by which time only 0.1 to 0.3 ml had been taken up by each plant (allowing for evaporation through the cotton wool plug). Under similar conditions it was found that aqueous dye solutions such as acid fuchsin or light green were rapidly absorbed into the vascular system, the whole plant being pervaded within a few hours. This absorption, however, represented a very small volume. Leopold and Thimann report that their solution (viz. 1 ml) was " generally entirely taken up by the plant within seven days."

Both the spring and winter varieties of Petkus rye were used. Experimental plants of the winter variety comprised two vernalised (10 weeks and 4 weeks at low temperature) and an unvernalised series. Vernalisation was carried out by the methods described by Purvis and Gregory (11). Each series was divided between long-days and short-days. The winter rye was first injected 3 weeks after the time of sowing and further injections were carried out after successive intervals of three weeks as long as the plants remained in a vegetative condition. All plants that had not reached the stage of ear-emergence 19 weeks from sowing were dissected after this time.

The spring rye was grown for 3 weeks in short-days; it was then injected and transferred to long-days. These plants were dissected after a further 3 weeks, that is, when they were 6 weeks old.

The plants were grown in sand culture in 10-inch pots with nutrients as described by Purvis and Gregory (10). Short-days (10 hours) were provided by the use of light-proof covers; long-days by the extension of the natural daylength when necessary to 17 hours by artificial light of low intensity (20 fc).

Experiments with Wintex Barley: In 1951 and in the following two years, experiments were carried out with barley, variety Wintex. The experimental procedure followed exactly that described above for spring rye: seedlings were first grown for 3 weeks in short days. Dissection of plants after this time revealed that they were in a vegetative condition. They were then transferred to 17-hour days and injected with auxin solution. Five days after injection, 0.2 to 0.5 ml of solution had been absorbed by each plant. It will be noted that the absorption rate was higher in the barley than in the rye. The plants were dissected when they were 6 weeks old, that is 3 weeks from the time of injection.

Results

Experiments with Petkus Rye: Two concentrations of α-naphthaleneacetic acid were tested, a low concentration (0.01 mg/l) and a high concentration (30 mg/l). Neither of these concentrations influenced the time to flowering, the number of flower primordia, the leaf number, or the rate of tillering for each series of plants. No data for these experiments will therefore be presented.

Experiments with Wintex Barley: The results of two experiments with Wintex barley carried out in the summer of 1951 are presented in Table I. Replication in the first experiment was 15 plants and in the second experiment 10 plants per treatment. Variability is expressed as the standard error of the mean. The auxin solutions tested were from a series of dilutions commencing with a saturated aqueous solution of α-naphthaleneacetic acid at 20°C which is approximately 500 mg/l. The figures given in the table for the concentrations of auxin are therefore approximate.

Flower primordia are defined here as the initial or ridge at each node that gives rise to a group of 3 spikelets. Counts of flower primordia were confined to the main axis of each plant. Every concentration of auxin with the exception of the highest used (approximately 500 mg/l) increased the number of flower primordia over that of the controls. Some of the differences are statistically significant with a value of P lower than 0.01. The highest concentration reduced the number of flower primordia in the second

TABLE I

THE EFFECT OF INJECTED AUXIN (α-NAPHTHALENEACETIC ACID) ON THE NUMBER OF FLOWER PRIMORDIA, SPIKE LENGTH, AND TILLERING OF WINTEX BARLEY

CONCENTRATION OF NAA (APPROX. MG/L)	NUMBER OF FLOWER PRIMORDIA PER PLANT	SPIKE LENGTH (SCALE UNITS)	NUMBER OF TILLERS PER PLANT
Sowing Date: 30/5/51 Replication 15			
500	22.3 ± .39	56.1 ± 3.4	6.5 ± .59
0.5	25.6 ± .61	104.5 ± 8.3	6.9 ± .39
0.1	24.4 ± .59	91.1 ± 8.7	6.5 ± .24
0.05	23.3 ± .51	80.9 ± 4.3	7.1 ± .22
0.01	23.3 ± .60	84.6 ± 5.7	6.4 ± .24
Control	21.9 ± .46	64.5 ± 3.2	6.0 ± .41
Sowing Date: 17/6/51 Replication 10			
500	22.6 ± .86	32.4 ± 2.7	5.4 ± .82
50	30.1 ± .61	46.4 ± 2.0	5.9 ± .82
5	29.5 ± .65	42.1 ± 2.1	5.4 ± .48
0.5	31.2 ± .42	46.1 ± 1.9	7.5 ± .40
0.05	29.8 ± .71	43.7 ± 2.2	6.0 ± .52
0.005	29.1 ± .48	44.2 ± 1.1	6.2 ± .42
Control	26.7 ± .40	39.0 ± 2.0	4.7 ± .52

experiment but not in the first. These changes were parallelled by similar changes in the length of the spike. Tillering was slightly increased by all concentrations.

In the experiments of Leopold and Thimann the changes in number of flower primordia were exactly parallelled by changes in fresh weight. In the experiments recorded here, similar but slight changes in fresh weight were found but none of the differences were statistically significant.

The increase in number of flower primordia following injection of auxin in low concentration is thus confirmed, although the magnitude of this and corresponding effects was much less than that obtained by Leopold and Thimann. The increase in number of flower primordia in the experiments recorded here averaged 10 to 15 % of the controls, and the decrease by the high concentration in the second experiment approximately 15 %. Leopold and Thimann obtained a 40 % increase with a concentration of 0.01 mg/l, while the reduction in number of primordia by their highest concentration (400 mg/l) approached 80 %.

Figure 1 is a photograph of the young ears that were dissected out of the plants that were used in the first experiment of 1951 (sowing date: 30/5/51). It can be seen that the ears from plants injected with low concentrations of auxin, especially concentrations of 0.1 and 0.5 mg/l, are at a more advanced stage in spikelet differentiation. The figures on the right of figure 1 show the relative sizes of the primordia as calculated by dividing the length of the ear by the number of initials present. These differences may be attributed to (a) earlier initiation of flower primordia and/or (b) a post-initiation increase in the rate of development of the spike. It must be emphasised that the ear at this stage has entered upon the exponential phase of the grand period of growth, so that if the observed differences were due entirely to earlier initiation, an acceleration of 2 or 3 days would fully account for the larger ears in the treated plants from this experiment.

In February of 1952 and in the same month of 1953 the experiments described above were repeated on a larger scale with a view to ascertaining the time of initiation of flower primordia by the dissection of periodical samples. In this way it could be decided whether auxin treatment either hastened initiation or merely stimulated the post-initiation development of the spike. In these repeated tests the solution was absorbed to the extent of 0.3 to 0.5 ml per plant in 5 days. The lower concentrations employed failed to increase the number of primordia but the high concentration reduced the number of primordia by 15 %. In all treatments flower primordia were initiated on the spike within a week after transfer to long-days. As the results of these two experiments failed to show any stimulating effect on either initiation or primordia number no data will be presented.

DISCUSSION

In considering the possible effects of auxin on flowering behaviour it is necessary to define the meaning in which "promotion of flowering" or "favourable to flowering" are used. The changeover from the vegetative to the reproductive phase is unequivocally indicated by the first appearance of flower primordia, and this critical stage in development is denoted by the term "initiation." The interval elapsing between the beginning of treatment and this point in development can be measured either in days or in terms of the plastochrone (leaf number). The advantage of the latter measure accrues from the fact that it is independent of the rate of differentiation at the growing point; thus the same number of plastochrones may demand different periods of time at different temperatures.

Conditions of "promotion of flowering" or "favourable to flowering" may on the other hand be assessed by the number of flower primordia laid down and it would appear that Leopold and Thimann have used this as a measure of the intensity of flower induction.

In the Gramineae the number of flowering ridges developed on the ear is not directly associated with flower initiation, nor is this number uniquely determined by the photoperiodic conditions. A positive correlation is found between spikelet number and the weight of the plant (as Leopold and Thimann have shown in barley) and a similar correlation is found between spikelet number and the age of the plants when flowers are initiated (2, 5, 6, 9). Increasing the level of nitrogen supplied in the nutrients results in larger and heavier plants with more numerous flower primordia, while the time to flower initiation is unaffected (5, 9). The time to initiation depends only on the conditions of light and temperature, while the

number of spikelets is influenced by these factors only indirectly (that is, only in so far as light and temperature determine the onset of the reproductive phase).

In view of this discussion, it is clear that the "relative abundance of flower primordia" does not provide a "measure of flower initiation" in barley, but may indicate an effect of auxin on post-initiation development of the ear. An effect on initiation could only be claimed if injected auxin caused initiation to occur in unfavourable photoperiods. Leopold and Thimann showed that if the plants were not transferred to long-days when they were injected with auxin (that is injected and maintained in the former regime of short-days) the plants were still vegetative at the time of dissection. Auxin alone therefore does not hasten flower initiation in barley; at best it might enable the plants to respond more readily to long photoperiods. But since after transfer to long photoperiods, initiation is manifest within a week when no auxin is applied, any enhancement in the response brought about by auxin can only be small, at the most a few days acceleration. Such a slight improvement is suggested from the results of the 1951 experi-

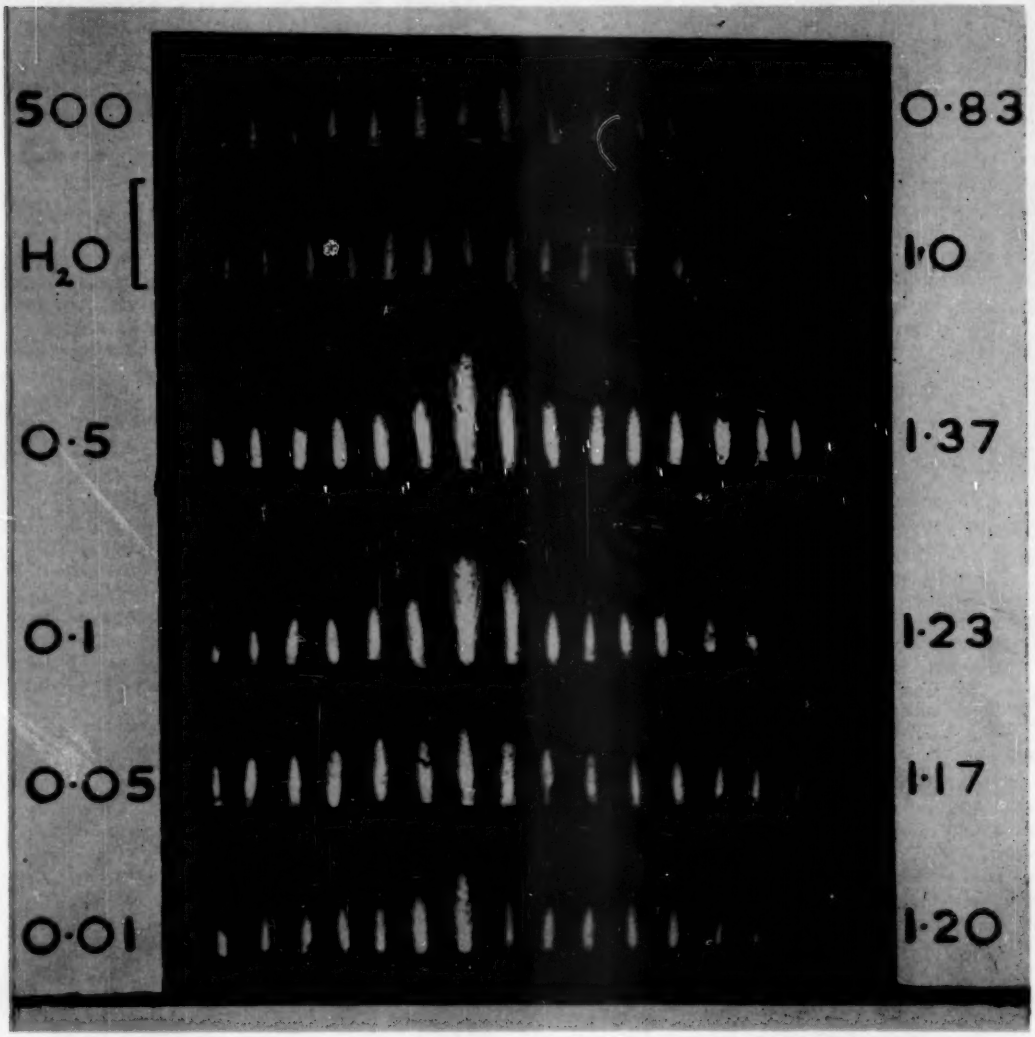

FIG. 1. Photograph of the young ears that were dissected from the Wintex barley plants used in the first experiment carried out in 1951, sowing date: 30/5/51. The figures on the left give the concentration of auxin solution injected (see table) and the figures on the right the relative sizes of the flower primordia. The scale printed on the left of the water controls equals 1 cm.

ments reported here, but as already pointed out, these results could also be accounted for on the basis of a purely post-initiation effect. In two experiments out of four carried out with Wintex barley no stimulating effects of auxin were found.

Summary

Plants of Petkus rye and Wintex barley were injected with auxin (α-naphthaleneacetic acid) according to the methods used by Leopold and Thimann for Wintex barley. The flowering behaviour of Petkus rye was not affected by the auxin. In two experiments out of four carried out with Wintex barley, auxin in low concentration increased the number of flower primordia while high concentrations decreased the number of primordia. This result, obtained in two experiments, confirms the findings of Leopold and Thimann, although the magnitude of the effects was much less in the experiments reported here.

It is suggested that the results of Leopold and Thimann may be evidence for a post-initiation effect of auxin on the developing ear, and in this sense may be said to promote or inhibit flowering, but there is no evidence either in the experiments of Leopold and Thimann or those reported here, that auxin has any determinable effect on flower initiation.

The authors wish to thank Mr. W. G. MacKenzie, Curator of the Chelsea Physic Garden where this work was carried out, and Dr. Erwin Spear who kindly supplied the seed of Wintex barley.

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BRIEF PAPERS

THE ELECTROPHORETIC BEHAVIOR OF LIGNIN[1,2]

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The precipitation of lignin from aqueous solutions of sodium or potassium hydroxide is rather easily brought about by lowering the pH to from 3 to 4, but is not effected by the addition of organic dehydrating agents. The observation that under definite conditions a gel can be formed has led to a consideration of its electrophoretic behavior.

At the pH levels at which lignin is soluble, the charge on the complex is probably due to the dissociation of phenolic hydroxyl groups together with enolizable carbonyl groups. At the pH of most soils, however, conditions are not only unfavorable for the ionization of the phenolic hydroxyls but often cause the precipitation of isolated lignin. It is over this pH range, however, that lignin undergoes various reactions, ultimately forming humus.

Schubert et al (1) have investigated the electrophoretic activity of soluble native and of enzymatically liberated lignins over the pH range of approximately 9.5 to 11. Both lignins increased in mobility as the pH increased, with considerable mobility at the zone of initial precipitation. The electrophoretic pattern for oak lignin indicated a homogeneous compound, and the pattern for white Scots pine indicated a heterogeneous compound.

The purpose of this investigation is to determine the electrophoretic behavior of lignin and modified lignins over the pH range of about 2 to 6.

METHODS AND MATERIALS

The lignin samples tested were: (a) alkali lignin, prepared from corncobs, purified by alcohol, acetone, and electrodialysis; (b) hydrated lignin, prepared by precipitating corncob lignin from solution in sodium hydroxide upon sufficient acidification followed by dialysis; (c) methylated lignin, obtained by methylating Meadol[3] with dimethyl sulphate in sodium hydroxide until the methoxyl content was approximately 34 %; (d) oxidized lignin, prepared by passing oxygen through a solution of Meadol in concentrated ammonium hydroxide for about 150 hours (2). The oxidized product contained approximately 7.2 % nitrogen, 33 % of which could be removed by distillation with magnesium oxide.

The dry products were suspended by flushing repeatedly one gm of material with 300 ml of water through a manual homogenizer of minimum orifice. Fifteen ml of the uniform suspension and 5 ml of buffer were transferred to a 100-ml volumetric flask and made to volume. The ionic strength of the final suspension was standardized at 0.045.

The electrophoretic apparatus was a calibrated Abramson microelectrophoresis cell, placed in a brass frame mounted on the stage of a Zeiss microscope. A 10× occular and "C" objective were used to give a magnification of about 220. A 100-watt bulb furnished the light, the rays of which were passed through a one liter round-bottom flask containing a dilute solution of copper sulphate. Electric current was measured with a milliammeter and controlled by means of a rheostat. Movement of the particles was controlled by means of a double-pole double-throw toggle switch (3). Particles were allowed to travel a total distance of 0.112 mm, 0.056 mm each way, as determined on an eyepiece micrometer scale; the 0.79 level was used for observations. The time consumed in migration was measured to one fifth of a second with a stop watch; ten measurements were made for each pH. The average was considered representative of the mobility of the particle for the existing conditions. Prior to each test the cell was flushed out thoroughly with the test suspension. Conductivity measurements were made at 25°C with a 1000-cycle Wheatstone bridge conductivity apparatus equipped with a 6 E 5 electron ray tube.

RESULTS AND DISCUSSION

The data obtained are illustrated graphically in figure 1. The most outstanding observations are (1) the uniformity of the slope of the lines relating mobility and pH and (2) the fact that all substances bear a negative charge. Below pH 2.5 the movement was too slow to be timed readily; evidence of a reversal of charge was not obtained. The mobility at the higher pH levels was enhanced considerably. The charge on the particle in all these cases is believed to be due to adsorption of an ion from the dispersion medium. The fact that the mobility data for the unaltered lignin are very similar to those for the methylated product would indicate that the phenolic hydroxyls play no direct part in the origin of the charge.

When purified lignin is hydrated there is an increase in the surface and in the number of groups exposed which is reflected in an increased mobility; this is substantiated by the data shown.

The oxidized lignin contains a rather firmly bound nitrogen atom. Since only lignin products with two or more phenolic hydroxyls were found to be significantly susceptible to the simultaneous oxidation and nitrogen fixation process, it has been assumed that the reaction took place via the phenolic hydroxyls (4), which probably were inactivated. The general result

[1] Received August 24, 1953.

[2] Contribution No. 909 of the Massachusetts Agricultural Experiment Station.

[3] Kindly furnished by the Mead Corporation, Chillicothe, Ohio.

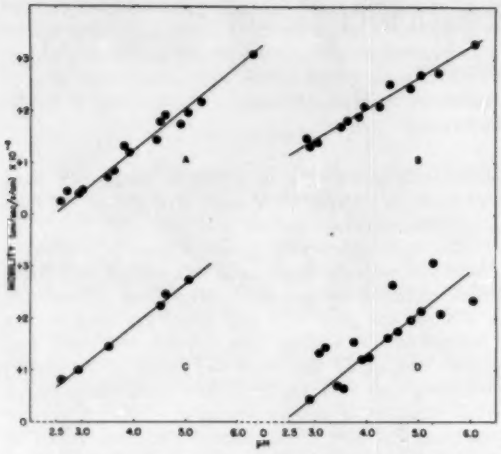

FIG. 1. The effect of pH on the electric mobility of: (A) purified corncob lignin, (B) ammoniated and oxidized Meadol, (C) hydrated corncob lignin, and (D) methylated Meadol.

of the simultaneous reaction was that the mobility was enhanced over that of pure lignin.

The fact that the oxidized lignin and the hydrated lignin have similar mobility rates, although the oxidized lignin was definitely dehydrated prior to testing, might indicate that the simultaneous reaction exposes a group or groups that are also exposed when hydrated. It should be remembered that the oxidation occurred when lignin was essentially in solution. The obvious conclusion, however, would be that the enhancement of mobility resulted directly from the simultaneous reaction. The exact nature of this reaction is not clear. Mattson and Koutler-Andersson (4) have suggested that the nitrogen might be present as quinone imides, thus accounting for the high ammonia fixation. In this investigation there is no evidence to indicate that the oxidized lignin is amphoteric over the pH range used. Nevertheless, it is notable that the charge on the compounds over the pH range 2 to 6 is negative, as reported by Schubert et al (1) for certain lignins in the upper pH range.

SUMMARY

The electrophoretic behavior of modified lignins was determined in buffered solutions of constant ionic strength over the pH range 2 to 6, by means of the Abramson cell. The rate of increase of mobility as a function of pH was relatively constant; there was no evidence to indicate that the products were amphoteric. The particles were negatively charged.

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DIPHOSPHOPYRIDINE NUCLEOTIDE-NITRATE REDUCTASE FROM SOYBEAN NODULES[1,2]

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The properties of a nitrate reductase from soybean leaves, which catalyzes the reduction of nitrates to nitrites by the reduced derivatives of either diphosphopyridine nucleotide (DPNH), or triphosphopyridine nucleotide (TPNH) was recently reported (3). A similar nitrate reductase exhibiting a striking specificity for TPNH was partially purified from *Neurospora crassa* (2, 8). Both enzymes are flavoproteins with flavin-adenine dinucleotide as the natural prosthetic group. The activities of the enzymes were similarly inhibited by certain metal chelating compounds and by sulfhydryl complexing agents. The pH for optimum activity of the higher plant enzyme was 6.0 and that of the Neurospora enzyme was 7.0.

It is the purpose of this communication to report results of initial experiments concerning certain properties of a highly specific DPNH nitrate-reductase from nodules of the soybean plant.

MATERIALS AND METHODS

Nodules were obtained from soybean plants (*Glycine max* Merr., variety Ogden) grown in sand cultures without combined nitrogen (3) and also from soybean and other leguminous plants (table I) growing in fields near Raleigh. Soybeans grown in sand cultures were inoculated with a commercial preparation containing *Rhizobium japonicum*, which was obtained from the Albert Dixon Company of Chicago, Illinois. Immediately after removal from roots, the nodules were placed in crushed ice and all preparatory

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[2] Contribution from the Division of Biological Sciences, North Carolina Agricultural Experiment Station, and published with the approval of the Director as paper No. 509.

operations thereafter were carried out at 0 to 4°C. Homogenates were prepared by grinding with a cold mortar and pestle 1 weight of nodules with 3 weights 0.1 M potassium phosphate buffer at pH 7.0. Cell free extracts of soybean nodules collected in the field were prepared as follows: Homogenates were centrifuged at 20,000 × g for 15 minutes and the supernatant liquid containing the hemoglobin was collected. The sediment was suspended in the original volume of 0.1 M K_2HPO_4 buffer at pH 9.0 and ground in a Ten Brock homogenizer for 30 minutes, centrifuged at 20,000 × g for 15 minutes and the supernatant fluid collected and used as the crude extract. Washed bacterial cells were prepared by suspending the sediment from the first centrifugation of the soybean nodule homogenate in 0.1 M phosphate buffer at pH 7.0, centrifuging at 600 × g for 5 minutes, discarding the precipitate, then collecting the cells in the supernatant fluid by centrifugation at 20,000 × g for 15 minutes. Microscopic examination of this material revealed an almost pure suspension of bacilli.

Sufficient ammonium sulfate was added to the crude extract to obtain 25 % saturation. The precipitate was discarded and additional ammonium sulfate added to obtain 48 % saturation. The precipitate obtained was dissolved in 30 % of the original volume of 0.1 M phosphate buffer at pH 7.0. This fraction is referred to as the 25 to 48% ammonium sulfate precipitate. It was further purified by adsorption upon and elution from calcium phosphate gel as previously described (3). It was necessary, however, to carry out the adsorption procedure twice before an appreciable amount of enzyme was obtained on the gel.

The preparation of reduced coenzymes and the procedure for nitrate reductase assays have been described (3). The methods used in the experiments reported here were the same with the exception that hydroxylamine hydrochloride was omitted in reaction mixtures containing homogenates. All TPNH and DPNH preparations were shown to be enzymatically active with purified soybean leaf nitrate reductase (3) prior to their use with the nodule preparations. The protein contents of cell free extracts were determined by the method of Lowry et al (5) and those of homogenates were determined by total N analyses × 6.25.

Results and Discussion

The high specificity of the enzyme for DPNH is shown by the activities of cell free extracts with the different reduced coenzymes (table I). When reaction mixtures contained equivalent concentrations of TPNH and DPNH (1.6×10^{-4} M, which was sufficient for maximum activity with DPNH) the rate of nitrate reduction with TPNH was less than 10 % of the rate with DPNH. Increasing the TPNH concentration five-fold resulted in only slight stimulation of activity. As indicated in table I flavin-adenine dinucleotide was included in all assays. Its omission from mixtures containing the most purified fraction resulted in 10 % reduction in activity. This suggests that this enzyme is also a flavoprotein.

Further study of the properties of the partially purified enzyme showed that the pH for optimum activity was 6.5 to 7.5. A concentration of 10^{-2} M and 10^{-3} M potassium cyanide in reaction mixtures inhibited the activity 83 % and 4%, respectively. Sodium azide concentrations of 10^{-3} M and 10^{-4} M inhibited 81 % and 38 %, respectively, and 10^{-4} M cupric sulfate inhibited 41 %.

Table I

Nitrate Reductase Activity of Extracts and Homogenates of Nodules from Leguminous Plants *

Enzyme preparations	No. of samples	Nitrite formed in 10 minutes (means)		
		Without coenzyme	With DPNH	With TPNH
		$\mu M \times 10^3$ per mg protein		
Extracts of soybean (*Glycine max* Merr.) nodules				
Crude K_2HPO_4 extract	4	0.9 ± 0.5 **	15.4 ± 5.0	1.3 ± 0.8
25 to 48 % $(NH_4)_2SO_4$ precipitate	4	0.4 ± 0.2	30.0 ± 7.1	2.1 ± 0.7
Calcium phosphate gel eluate	2	0.0 ± 0.0	76.0 ± 7.3	3.0 ± 1.3
Nodule homogenates				
Soybeans (field grown) ***	4	69.0 ± 9.3	73.0 ± 13.3	71.0 ± 5.1
Soybeans (sand cultures without combined N) ***	2	55.0 ± 5.8	64.0 ± 7.7	63.0 ± 6.1
Bacteria from homogenates of soybean nodules (field grown) ***	2	202.2 ± 16.3	240.0 ± 17.8	199.1 ± 15.2
Peanuts, *Arachis hypogaea* L. (field grown)	2	1.6 ± 1.2	13.9 ± 2.3	....
Lespedeza stipulacea Maxim. (field grown) †	2	3.0 ± 0.7	3.9 ± 0.9	2.8 ± 0.6
Lespedeza bicolor Turcz. (field grown) †	1	4.8 ...	6.8 ...	5.2 ...

* Reaction mixtures contained: 10 μM of $NaNO_3$; 50 μM of phosphate buffer at pH 7.0; 10^{-3} μM of FAD; 0.08 μM of DPNH or TPNH as indicated; and aliquots of homogenates containing 0.9 to 2.5 mg protein, or aliquots of extracts containing 0.7 to 5.0 mg protein. The final volume was 0.5 ml.

** Standard deviation.

*** Reaction time of 5 minutes, results calculated on a 10 minute basis.

† Reaction time of 30 minutes, results calculated on a 10 minute basis.

"Nitrite reductase" assays (3) on ammonium sulfate fractions and calcium phosphate gel eluates showed no nitrite disappearance during 10-minute incubations. Homogenates, however, consistently exhibited "nitrite reductase" activity.

Assays of nodule homogenates from four different leguminous species (table I) indicate that a nitrate reductase is present in all samples tested. It is interesting to observe the high activity of nodule homogenates from plants grown in sand cultures without combined nitrogen. The high specific activity of the washed bacteria is strong evidence that the enzyme is associated with *Rhizobium* cells rather than higher plant cells. This conclusion is supported by the fact that the original supernatant liquid from the homogenate (containing 10 mg protein per ml and practically all the hemoglobin from the nodules) was completely devoid of nitrate reductase. It was essential to grind the bacterial cells in alkaline buffer in order to obtain the enzyme in solution.

The results with crude homogenates do not permit definite conclusions regarding coenzyme specificity—but those with cell free extracts of soybean nodules are clear. By the use of acetone powders of soybean nodules some evidence was obtained that a weak TPNH nitrate reductase was present. These experiments however require further confirmation.

Perhaps it is worthy of note that washed cells from a pure culture of *Pseudomonas solanacearum* E.F.S. (kindly supplied by Dr. Arthur Kelman) exhibited a very high nitrate reductase activity which was markedly stimulated by either TPNH or DPNH.

The most obvious interpretation of the physiological role of nitrate reductase in the nodules of leguminous plants is its probable involvement in the metabolism of nitrates that enter nodules under field conditions or perhaps enter nodules from impurities in nutrient salts supplied to plants grown in sand cultures. Neither nitrates nor nitrites are seriously considered as theoretical intermediates in nitrogen fixation by symbiotic bacteria (10) but hyponitrite has been postulated as a first product of fixation assuming that the initial fixation reaction is oxidative rather than reductive. In view of the unstability of hyponitrites the possibility of producing nitrates and nitrites from hyponitrites by oxidation does not seem unreasonable.

It was recently demonstrated (9) that molybdate was an essential constituent of the nitrate reductase from Neurospora. And in view of the similar inhibitory characteristics of the nitrate reductases from Neurospora (8), soybean leaves (3), and soybean nodules, and the fact that molybdenum deficiency in either higher plants or fungi (7) results in nitrate accumulation, it seems highly probable that molybdate is essential for all three nitrate reductases. It is well established that molybdenum is necessary for the utilization of nitrates by legumes (1, 6) and that an increased supply is required when leguminous plants are forced to utilize atmospheric nitrogen. Jensen (4) has reported that nodules from alfalfa contain 5 to 15 times higher concentrations of molybdenum than roots of plants. These observations have been confirmed by analyses in our laboratory. Also molybdenum analyses of various fractions of peanut and soybean nodules have revealed that suspensions of bacterial cells contained 2 to 3 times greater concentrations of molybdenum per mg of protein than hemoglobin extracts of nodules which were devoid of nitrate reductase. In relation to the inhibition of nodule nitrate reductase by cupric salts it is interesting to consider experimental results (unpublished experiments by the author) with inoculated Ladino clover grown in pot cultures of soil without combined nitrogen and with various levels of copper and molybdenum. Fifty pounds of cupric sulfate per acre reduced yields to one third that of controls but the same amount of cupric sulfate with 5 pounds of sodium molybdate per acre caused no significant reduction in yield.

There seems to be sufficient indirect evidence to consider the possibility of a role of nitrate reductase in nitrogen fixation as well as in nitrate metabolism. It is necessary to postulate an oxidative step as the initial reaction in nitrogen fixation in order to visualize a possible role of this enzyme in the nitrogen-fixing process.

Summary

Cell free extracts of soybean nodules have been prepared which catalyze the reduction of nitrates to nitrites in presence of reduced diphosphopyridine nucleotide. The enzyme activity is strongly inhibited by sodium azide and cupric sulfate and weakly inhibited by potassium cyanide. Initial purification procedures have resulted in a five-fold increase in specific activity. It was shown that nitrate reductase is present in all of the eleven samples of nodules tested, from four different legume species. Results are discussed from the standpoint of the requirement of molybdenum for both nitrate reduction and nitrogen fixation, and it is concluded that there is ample indirect evidence to consider the possibility of a role of nitrate reductase in nitrogen fixation.

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POSSIBLE INTERACTION BETWEEN LIGHT-DARK CYCLES AND ENDOGENOUS DAILY RHYTHMS ON THE GROWTH OF TOMATO PLANTS [1]

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In the course of an experiment in which plants were grown under various alternations of light and darkness, it was found that some plants grown under conditions which deviated too greatly from a 24 hour cycle of 12 hours light and 12 hours darkness were inhibited in their growth.

Sunflowers, peas, and tomatoes were grown from seed under cycles of 6 hours of light–6 hours of darkness, 12 hours of light–12 hours of darkness, and 24 hours of light–24 hours of darkness. The plants were grown in vermiculite at 23°C and about 1000 fc of artificial light from warm white fluorescent lamps supplemented with light from incandescent bulbs. In a 48 hour period all plants received the same quality and quantity of light.

The most striking effects were those on the growth of the tomato plants (fig 1). The peas showed some inhibition of growth in the 6–6 and the 24–24 hour regimes as compared to the 12–12 hour treatment, but the sunflowers were not significantly affected. Garner and Allard (3) have shown that short alternations of light and dark inhibit the growth of many plants. Arthur, Guthrie, and Newell (1) found that tomato plants were extremely sensitive to long day length and will not survive under continuous illumination. The leaf injury symptoms reported by these authors are similar to those observed here—small, stiff, yellow leaves with dark necrotic spots. In addition it was noted that the number of nodes per plant was not significantly reduced by the abnormal light-dark treatment. Arthur et al found that the injury to the tomato plants was not due to an excessive accumulation of carbohydrates, and considered it to be due to a breakdown of the photosynthetic process. However, the results reported here were obtained with plants receiving equal quantities of light in every 48 hour period so that if photosynthetic mechanisms are involved, they must be sensitive to some superimposed periodic phenomenon.

Bünning (2) has formulated an hypothesis that the endogenous daily rhythm of plants determines such photoperiodic responses as flowering. He has proposed that during one phase (the photophile) of the endogenous daily rhythm, light promotes the assimilatory activities of the plant, such as those that affect flowering. At the other extreme of the endogenous daily rhythm, there exists a phase (the scotophile) in which light has no promoting effect or is even inhibitory.

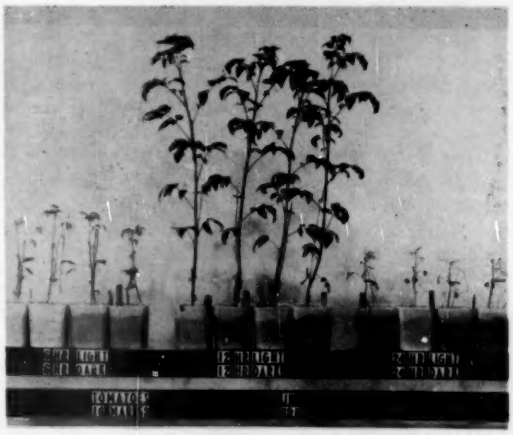

Fig. 1. The effect on the growth of tomato plants of 12, 24, and 48 hour cycles of alternating light and dark periods.

The effect which light-dark periodicity has on the growth of tomato plants might well be explained on the basis of Bünning's hypothesis. Thus plants grown in the 6–6 and the 24–24 hour regimes would receive light during all or some portion of the scotophile phase, if the tomato has an endogenous daily rhythm of 24 hours (fig 2). That the tomato does have such a rhythm is shown by such a phenomenon as diurnal rhythms in bleeding (4). Continuous light should have the same effect as the alternation of light and dark periods in the 6–6 and the 24–24 hour regimes, since any light period in excess of about 16 to 18 hours

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would expose the plants to light during advanced stages of the scotophile phase.

If Bünning's hypothesis is applicable to the growth of some plants as well as to such a phenomenon as flowering, a light interruption during the normal dark period (the scotophile phase) should be inhibitory. An experiment was designed to examine this point. Tomato plants were grown at a constant temperature of 23°C, at high intensity greenhouse light for 8 hours, from 8 A.M. to 4 P.M., followed by supplementary, artificial light of 1000 fc from 4:00 P.M. to midnight.

After initial growth rates were determined, half of the plants were given their supplementary light from 4 P.M. to 10 P.M. and from 2 A.M. to 4 A.M., thus interrupting the dark period with 2 hours of light. Both the control and the treated plants continued to receive a total of 16 hours of light of the same quality and intensity, with the night interruption being the only variable.

The results of this experiment are illustrated in figure 3. Dry and fresh weights fully confirm the inhibition shown by height measurements. Although highly significant statistically, the inhibition is not as great as expected. This confirms the observations of Arthur et al that plants grown in the greenhouse during the day are more resistant to the inhibitory effect of artificial light given during the night than are plants grown entirely in artificial light. A repetition of the dark interruption experiment with the plants growing entirely in artificial light of about 1400 fc and a constant temperature of 23°C shows a far greater inhibition of growth.

Bünning's hypothesis, although it gives us no mechanism to test, might be applicable to growth of plants such as tomatoes, which are photoperiodically indeterminate with respect to flowering, as well as to flowering of photoperiodically sensitive plants.

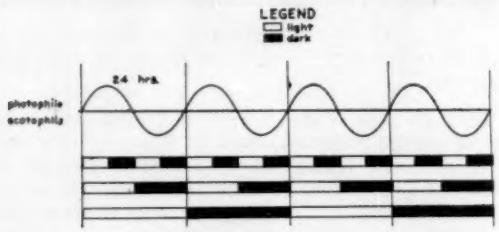

FIG. 2. Photophile phase: Phase of endogenous daily rhythm in which light promotes assimilatory activities of the plant. Scotophile phase: Phase of endogenous daily rhythm in which light is inhibitory or has no promoting effect.

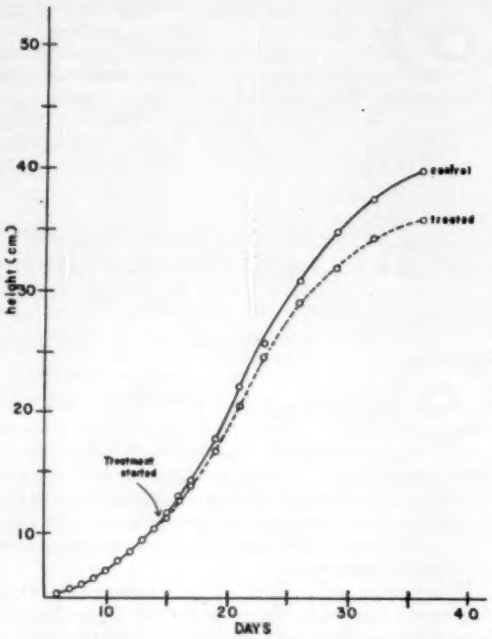

FIG. 3. Growth curve showing the effect of 2-hour light interruption of the normal dark period. The control plants received continuous light for 16 hours, and 8 hours of darkness. The treated plants received 14 hours of continuous light with a 2-hour light interruption of the dark period.

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METABOLISM OF 2,4-DICHLOROPHENOXYACETIC ACID. III. METABOLISM AND PERSISTENCE IN DORMANT PLANT TISSUE [1]

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In contrast to actively growing, susceptible plants which usually respond very promptly to applied 2,4-dichlorophenoxyacetic acid (2,4-D), certain perennials that have been treated toward the end of a growing season may not exhibit morphological responses until the following season (9, 10, 12, 13). It has been a moot question whether (a) such delayed responses are initiated in sensitive cells of winter buds at time of application of the growth-regulator but become visible only much later when the buds renew development or (b) 2,4-D, or some physiologically active product thereof, persists in the dormant plant and interacts the following season with tissues which become sensitive as they renew development (2, 3, 11, 13, 14).

Previous experiments with C^{14}-labeled 2,4-D have furnished direct evidence of relatively long persistence of the compound in bean plants. Although a portion of the applied growth-regulator is rapidly transformed into a variety of products (5, 15, 16), a significant amount may remain unaltered for at least 42 days (6). Less direct evidence of persistence of an active growth-regulator has been provided also by observations of protracted morphological responses in cotton (4, 7, 8) and of long delayed fruit abscission in apple (10). In these instances it has not been established whether the delayed or protracted responses are due to 2,4-D itself or to a biologically active transformation product.

The present experiment was undertaken with the objective of obtaining direct information concerning the metabolism and persistence of 2,4-D in dormant buds.

EXPERIMENTAL PROCEDURE

2,4-D labeled with C^{14} was applied to dormant buds of potted two-year-old cherry trees (*Prunus avium*) at the end of the second growing season. Half of the plants were treated on September 16, when leaves were still present, and half were treated on November 25, after abscission of all leaves. Separate lots of plants were treated with carboxyl-labeled, methylene-labeled, or ring-labeled 2,4-D, each form of the growth-regulator being applied at each of two doses of approximately 125 μg and 250 μg per bud. Each compound was applied to the five largest buds on each of two plants.

The compounds were applied by placing two or four 0.0025 ml drops of a 25,000 ppm acetone solution on each bud, allowing the surface of the bud to become dry after each addition. Very little of the solution ran off onto the adjacent stem. A considerable portion of the applied 2,4-D was not immediately absorbed through the bud scales and remained as a visible deposit on the surface. In order to allow time for penetration and to avoid possible loss from rain, the plants were taken into the greenhouse at time of treatment and held there during the succeeding two weeks after which they were returned out of doors where they remained exposed to rain and snow throughout the winter. It was shown in a separate experiment that 10.6 % of the 125 μg dose and 20.6 % of the 250 μg dose could be recovered by thoroughly washing the exterior of the buds one week after application. The remainder is presumed to have penetrated into the bud.

On March 6, before any visible growth had begun, the treated buds, together with 2 cm of adjacent stem, were excised. The ten fragments constituting each sample were bulked, comminuted with sand in a mortar, transferred to extraction thimbles, and extracted for five three-hour periods with successive 100 ml portions of boiling 80 % ethanol. The extracts were made to volume and aliquots removed for determination of C^{14} as previously described (15).

After removal of the alcohol by distillation, the aqueous solutions were acidified to pH 1 with sulfuric acid and extracted repeatedly with ether. Aliquots of the ether extracts were taken for C^{14} analysis and the remainder was chromatographed on filter paper with a number of solvents.

RESULTS

Since the results were essentially the same for the three forms and two doses of 2,4-D, only the average values are presented. As shown in Table I, 3.0 to 7.7 % of the applied radioactivity was recoverable from the buds and adjacent tissue several months after application of 2,4-D-C^{14}. The buds treated in November contained more than twice as much C^{14} as those treated in September. More time was, of course, available for metabolism to volatile products or for export from the buds treated at the earlier date; furthermore these processes would be expected to be more rapid at the higher temperatures prevailing between the first and second applications than during the subsequent colder weather.

Even the very incomplete fractionation that was made of the extracts served to demonstrate that part of the 2,4-D was transformed into at least two other products. An appreciable fraction of the recovered C^{14} was in the form of ether-insoluble substances which it was not attempted further to separate. The ether-soluble fraction also comprised two substances which were separable by paper chromatography. The major component, which contained, on the average, 85 % of the total ether-soluble radioactivity, was identified as unaltered 2,4-D by its chromatographic behavior and by bioassay (1). Cochromatograms

[1] Received December 2, 1953.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN DORMANT CHERRY BUDS AT THREE AND FIVE MONTHS AFTER APPLICATION OF 2,4-D-C^{14} (EXP. 1481)

C^{14} FRACTION	PERCENT OF APPLIED C^{14}	
	TREATED 16 SEPT. 1952	TREATED 25 NOV. 1952
Ethanol-insoluble	0.6	1.3
Ethanol-soluble, ether-insoluble	0.6	1.8
Non-2,4-D acid	0.3	0.5
Unaltered 2,4-D	1.5	4.1
Total C^{14} present in bud	3.0	7.7

with pure 2,4-D gave a single spot at Rf 0.80 with 90 % aqueous *n*-butanol and at Rf 0.73 with 75 % aqueous phenol. The physiological activity was that expected for 2,4-D. The 2,4-D comprised approximately half of the total radioactivity present in the tissue at harvest.

DISCUSSION

These results demonstrate that 2,4-D may undergo certain transformations in "resting" plant organs. At the same time a sufficient quantity of the compound may persist throughout the dormant period to induce morphological abnormalities when growth resumes the following season. It appears, therefore, that delayed responses such as have been observed to follow application of 2,4-D could be due to a reaction of the growth-regulator with cells that are formed long after the treatment as well as to delayed expression of injury induced at time of treatment.

The chromatographic evidence indicates that the same ether-soluble transformation product was obtained from the variously labeled 2,4-D molecules, showing that the carboxyl, methylene, and number 1 ring carbon atoms are all present, and suggesting that the molecule had not been extensively metabolized in the formation of this product.

SUMMARY

2,4-Dichlorophenoxyacetic acid applied in autumn to winter buds of cherry is in part metabolized to other products during the dormant season. A portion, however, remains unaltered until the following spring.

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THE EFFECT OF LIGHT INTENSITY ON PHOTOSYNTHETIC RATE IN SCOTCH PINE[1]

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Considerable ecological significance has been attached to comparative photosynthetic responses of tree seedlings to a range of light intensities (1, 2). Information regarding the response of Scotch pine seedlings, a species of importance in forestry, has not been available in the past and is presented in this report. An improved method is described for measuring photosynthetic rates of intact terrestrial plants.

METHODS

The apparatus, which was a modification of one previously described (3), is shown diagramatically in figure 1. The shoot of a seedling was enclosed in a chamber (*1*) consisting of a glass-topped cylinder of sheet iron which rested on a heavy brass plate (*2*). The chamber was assembled about the shoot, and all joints were sealed with modeling clay. Air within the chamber was circulated continuously by a small fan driven by a toy electric motor (*3*). Constant air temperature was maintained by passing cold tap water through copper tubing soldered to the wall and base plate. The flow of tap water was controlled by a thermoregulator (*4*) and solenoid valve.

The shoot chamber, an infra-red gas analyzer (*5*), and an air pump (*6*) were in a closed series connected with plastic tubing and ground glass joints. Air circulated continuously (1000 ml per minute) from the chamber, through the analyzer, and back to the chamber. The air pump was of the pulsating diaphragm type (a modified Model 1 Thiberg Aerator). The analyzer was a Model 15 Liston-Becker. The principle of the infra-red type analyzer and the history of its development have been reviewed recently by Parker (6).

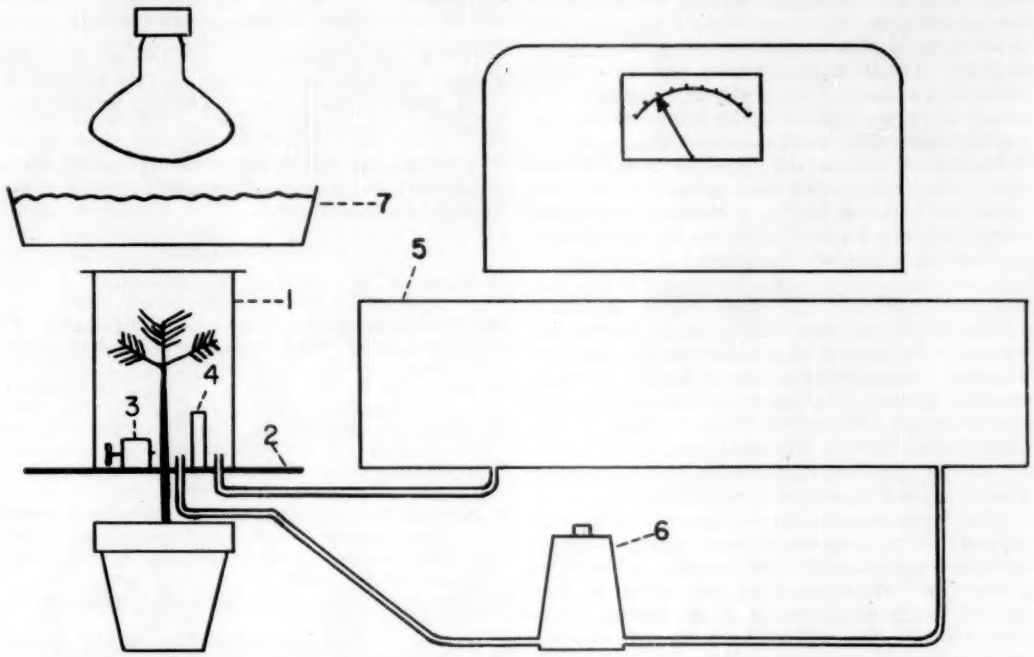

FIG. 1. Apparatus used to measure rates of apparent photosynthesis and respiration. Numbers refer to descriptions in text.

The gas analyzer was apparently similar to that used by Huber (4) and by Parker (7). It served as a direct-reading meter which, with a lag of approximately 5 seconds, indicated continuously the CO_2 concentration of the air enclosed with the plant. Reproducibility of readings was such that the overall accuracy of an analysis appeared to be limited by the writer's ability to calibrate the analyzer rather than by the precision and sensitivity of the analyzer itself. Calibration was done by injecting known quantities

[1] Received December 7, 1953.

of CO_2 into a closed, recycling system consisting of a 1000 ml flask, the pump, and the analyzer. Injections were made directly through thick-wall rubber tubing using a 20 gauge needle and a hypodermic syringe set to deliver 0.240 ml. Sensitivity drifted somewhat with changes in room temperature, but checking against a reference gas (a tank of compressed air) every 30 minutes was sufficient to hold deviations to within 1 %.

Illumination was provided by a battery of four 150-watt spotlights focused on the chamber through a filter (7) of cold, running water. The degrees of illumination were attained by covering the top of the chamber with different thicknesses of white paper.

The seedlings were approximately three years old (two years in seed beds, one growing season in pots).

The operating procedure was as follows. A seedling was sealed in the chamber and allowed to stand approximately 10 minutes under full light. A quantity of CO_2 was introduced into the system to raise the concentration slightly above 0.045 %. The time required for it to fall from 0.045 % to 0.034 % was recorded. The mean concentration was thus fixed at 0.0395 % and most of the problems of variable CO_2 supply (3) were avoided. The procedure was repeated twice. The illumination was then reduced to the next level, and the whole procedure was repeated. Respiration measurements were similar but were made in the dark and with the CO_2 concentration first being lowered slightly below 0.034 % by the introduction of a soda-lime tube into the system.

Results and Discussion

The results are shown in figure 2. Each point represents the mean of 18 observations (triplicates on 6 plants). Photosynthetic rate of Scotch pine seedlings was shown to be a linear function of illumination over the range 1800 to 6400 fc and it dropped more steeply below 1800 fc. The curve was similar to that for loblolly pine seedlings shown by Kramer and Decker (5) and reproduced here in figure 2.

The increased precision of the method becomes apparent when the spread of points about the curves in figure 2 is considered. The curve for loblolly pine is based on 7 observations per point using an older method. A manyfold increase in the number of observations would be required for the spread of points to approach that associated with the curve for Scotch pine. For example, to reduce the spread by 75 % would require approximately a 16-fold increase in the number of observations.

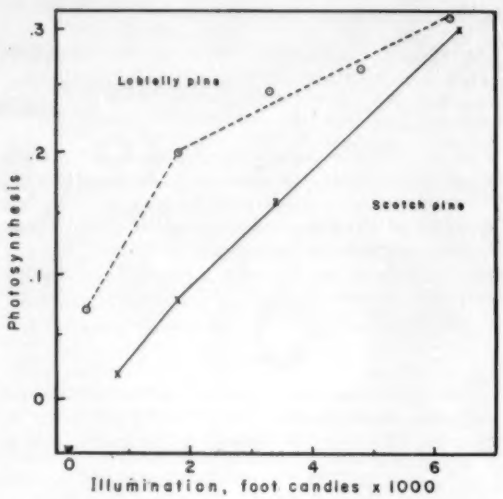

Fig. 2. Photosynthetic rate (mg CO_2 per shoot per minute) of Scotch pine seedlings vs. light intensity at 25°C. Data for loblolly pine (mg CO_2/10 $cm^2 \times hr$) were taken from Kramer and Decker (5).

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NOMENCLATURE OF CHEMICAL PLANT REGULATORS

REPORT BY A COMMITTEE OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

In 1951, K. V. Thimann, in his capacity as President of the American Society of Plant Physiologists, appointed a committee "to consider and propose a uniform nomenclature on growth substances."

The Committee consulted, on an international basis, over 200 fellow scientists with interest in chemical regulation and in nomenclature, and asked for their collaboration in the project through an expression of their views. The participation was general and most gratifying, involving considerable free discussion. The reactions and suggestions of the majority opinion have been incorporated in the committee's recommendations. Accordingly, the proposed nomenclature is necessarily one of compromise. Yet it is simple and necessitates a minimum of reorientation of thought, and also allows for expansion in the future.

The idea of artificial regulation of plant physiological processes by means of chemicals is a corollary to the concept of phytohormones which involves the regulation of plant physiological processes by naturally occurring regulators. The latter relates one organ to another in the plant. It follows therefore that the nomenclature on regulators is primarily applicable to multicellular plants.

Definitions

(Plant) Regulators are organic compounds, other than nutrients,[1] which in small amounts promote, inhibit or otherwise modify any physiological process in plants.

(Plant) Hormones (Synonym: Phytohormones) are regulators produced by plants, which in low concentrations regulate plant physiological processes. Hormones usually move within the plant from a site of production to a cite of action.

Growth Regulators (Synonym: Growth substances) are regulators which affect growth.

Growth Hormones are hormones which regulate growth.

Flowering Regulators are regulators which affect flowering.

Flowering Hormones are hormones which initiate the formation of floral primordia, or promote their development.

Auxin is a generic term for compounds characterized by their capacity to induce elongation in shoot cells. They resemble indole-3-acetic acid in physiological action.

Auxins may, and generally do, affect other processes besides elongation, but elongation is considered critical. Auxins are generally acids with an unsaturated cyclic nucleus or their derivatives.

Auxin Precursors are compounds which in the plant can be converted into auxins.

Anti-Auxins are compounds which inhibit competitively the action of auxins.

[1] Nutrients are here defined as materials which supply either energy or essential mineral elements.

The selection of the word *regulator* as a collective term for the biologically active materials under discussion was a difficult one. In several respects a term such as "ergon" or "ergocrine" might have been preferred. Yet the majority of the committee felt that to introduce a virtually new word into the literature might eventually lead to more confusion. It was thus decided to restrict the work of the committee to defining the boundaries of existing terms. Of the terms here discussed, *regulator* has the widest boundaries. It applies to materials that modify any plant physiological process. When in doubt about the more precise nature of a regulatory material, it is sufficiently identified by the word *regulator*. The term is especially recommended to replace the word "hormone" as it is used loosely, when referring to agricultural (or potentially agricultural) chemicals used for crop control. Naturally occurring regulators may be called *hormones* if they satisfy the requirements set forth under "Definitions." The survey showed clearly that the majority of biologists still restrict the term *hormone* to natural products. However, the word *regulator* is not necessarily restricted to synthetic compounds.

The term *auxin* is used as a group-name for compounds having similar physiological activity. In this usage the term finds analogy in "Estrogens," which likewise groups together compounds having like physiological—in this case estrogenic—activity. The selection of promotion of cell elongation as the critical characteristic of the auxins is admittedly arbitrary, but it is now firmly rooted in history and practice. The use of *auxin* as a collective name, rather than for one specific naturally occurring compound, has met with the complete approval of Kögl and of Haagen-Smit, discoverers of the chemical nature of the naturally occurring auxins, who together with the late F. A. F. C. Went coined the term. Specific auxins are identified by their chemical names.

Regulation may be further specified by adding to the word "regulator" the process it modifies: *growth regulator, flowering regulator,* etc. The term *growth regulator,* as recommended here, includes the auxins, but it is broader, and it encompasses other regulators that may modify growth. By analogy the word *growth hormone* is broader than naturally occurring

auxin. It may for instance include the B-vitamins known to be required for root growth, but primarily produced in the shoot.

It may be added that the terms considered here are not necessarily mutually exclusive. It is becoming apparent, for instance, that a compound can be an auxin as well as an anti-auxin.

The nomenclature here discussed was accepted by the Executive Committee of the American Society of Plant Physiologists at the Madison, Wisconsin, meeting, September 6, 1953. It is hoped that it will be universally accepted. Authors, editors, teachers, lecturers, etc. are likely to accept it because it represents basically the thinking of hundreds of scientists in the field. Many others will welcome any agreement which makes order out of the present confusion.

H. B. TUKEY, East Lansing, Michigan
F. W. WENT, Pasadena, California
R. M. MUIR, Iowa City, Iowa
J. VAN OVERBEEK (*Chairman*), Modesto, California

Note: Dr. Poul Larsen, a member of the above committee, has not signed the report, since he did not agree with the majority on all points.—The Editor.

NEWS AND NOTES

THE NEW ENGLAND SECTION: The New England Section of the American Society of Plant Physiologists met May 7 and 8, 1954 in Northampton, Massachusetts at the invitation of Smith College, under the chairmanship of K. E. Wright. A program of 15 papers was presented during the two day period. Following the dinner meeting Harriet B. Creighton gave an illustrated talk on Australia. Officers elected for the coming year are: Kenneth V. Thimann, Chairman; Hubert J. Dyer, Vice-Chairman; Linus H. Jones, Secretary-Treasurer.

THE SOUTHERN SECTION: The Southern Section of the American Society of Plant Physiologists met with the Association of Southern Agricultural Workers in Dallas, Texas, February 1–3, 1954. Two sessions of submitted papers were presented. Abstracts of all papers will appear in the 1954 Proceedings of the Association. At the traditional breakfast Dr. Walter E. Loomis gave an address entitled "Growth of Plants." The breakfast was well attended and the subject provoked considerable discussion. Officers for 1954–55 are: Chairman, Roger W. Bledsoe, Florida Agricultural Experiment Station; Vice-chairman, John B. Whitney, Clemson Agricultural College; Secretary-Treasurer, Wayne C. Hall, Texas A. and M. College System; Directors, P. J. Kramer, Duke University, E. M. Emmert, University of Kentucky, D. E. Davis, Alabama Polytechnic Institute, H. E. Hammar, U.S.D.A., Shreveport, Louisiana. The 1955 meeting is scheduled to be held at Louisville, Kentucky.

THE SURVEY OF THE PHYSIOLOGICAL SCIENCES: The Survey of the Physiological Sciences, sponsored by the American Physiological Society, is carrying out a comprehensive analysis of the present status and trends in physiological science throughout biology. The Survey covers animal, plant, and bacteriological physiology and is concerned with both the fundamental and applied aspects of the science. Efforts are being made to determine the extent to which physiology is taught and investigated in biology, medicine and agriculture, whether in academic, government, clinical, or industrial organizations. The interest of the Survey includes a consideration of the personnel, finances, organizational aspects, professional societies, policy-making and students.

The operations of the Survey are concentrated in a central office in Washington under the supervision of an Executive Director and are subject to the constant review and analysis by a Central Committee composed of recognized representatives from all phases of the physiological sciences. Among the techniques being used by the Survey are an extensive analysis of the existing literature, a thorough examination of existing personnel rosters and other published data, and a questionnaire and interview system to be used with over 7,500 professional physiologists.

The Survey earnestly solicits the cooperation of all physiologists in obtaining the information desired from which significant and far reaching decisions will surely be derived.

Suggestions and comments concerning areas of interest or specific problems which should be covered by the Survey will be most welcome from all physiologists.—L. M. N. BACH, Executive Director, 2101 Constitution Avenue, Washington 25, D. C.

GEORGE PLUMER BURNS

Professor Burns died November 13, 1953 after a long illness in his eighty-third year. He was one of the founders of the American Society of Plant Physiologists and of the Ecological Society of America, as well as Professor of Botany and Chairman of the Department at the University of Vermont for thirty-four years until his retirement in 1944. He was also a member of Sigma Xi, Phi Beta Kappa and Alpha Zeta.

After earning his B.S. and A.M. degrees at Ohio Wesleyan, he obtained his Ph.D. at Munich under Dr. Goebel in 1900.

On returning to this country he was appointed Assistant Professor of Botany at the University of Michigan and subsequently Director of the Botanical Gardens. His interest in ecological research was evidenced by a series of studies of Michigan peat bogs. At the same time his vision and breadth of interests

led to the founding of the biological station at Douglas Lake at Cheboygan and the initiation of a park system in Ann Arbor, Michigan.

His research and numerous publications were in ecology and physiological plant anatomy. After the peat bog studies in Michigan he initiated in Vermont a series of investigations on the tolerance of forest trees. These resulted in a number of publications in which he made important contributions to the literature on the effect of light (both quantitative and qualitative), temperature, soil moisture and spacing on the rate of tree growth. He investigated the problem of eccentric growth in stems and produced it experimentally. His interest in fundamental research on important problems stimulated his son to work in photosynthesis.

Dr. Burns was for many years a leader of the faculty and on several occasions served as acting Dean of the College of Agriculture and Director of the Vermont Agricultural Experiment Station. More important, he was an inspiring teacher both to undergraduate and graduate students and because of his interests in students as well as his insistence upon high standards of academic achievement, he directed the graduate program of the University for many years. At the same time, he acted upon his sense of responsibility to the community of which he was a part. For twenty-five years he served as park commissioner for the City of Burlington and also as Superintendent of Parks, and the city benefits today from a bathing beach, athletic fields and play grounds added through his efforts.

While at the University he taught elementary botany and plant physiology, and was a master of the Socratic method kindly but always rigorous in the intellectual standards he required of his students. To quote from a University Senate resolution: "Without him we could not easily account for much of the strength and vigor we find in the University today."

Dr. Burns was born October 30, 1871 in Maroa, Illinois, the son of George M. and Emily Mouser Burns. He is survived by Mrs. Burns, two daughters and a son, and five grandchildren.—JAMES W. MARVIN.

THE CULTIVATION OF ANIMAL AND PLANT CELLS.—Philip R. White. The Ronald Press Company, 15 East 26th Street, New York, New York. 713 pages, 1954. $6.00.

When I was asked to review White's manual on tissue culture, I fully expected the task to be a dull one, for a laboratory manual is usually a mere schedule of what to do next and therefore unavoidably dull. My fears were not helped by the page at which I first opened the book, for there was a plan of the ideal laboratory, preparation room, sterilization chamber, and office, nothing being said about where the funds for building such a luxury are to be had. I then set about my task by starting at page 1, where there is a chart of the history of cell culture commencing with Aristotle and Theophrastus whose writings on the parts of animals and plants are, I presume, the reason for regarding them as the forerunners of the study of cells. Schleiden and Schwann follow, then Claude Bernhard. With tissues, cells, and a *milieu interieur*, we have all that Haberlandt and Harrison had for the first experimental attempts at tissue culture.

On page 2 is the Dedication, "to all those restless spirits" in science; I now knew that the book was not going to be dull and that it would be more than a manual. In both respects I was agreeably, at times surprisingly, pleased.

The foreword tells us that "tissue culture" is a misnomer, and to say "cell culture" is equally bad. What one really cultivates are groups of cells from tissues, never tissues, nor individual cells. This leads to the statement that all true cells are parts of tissues, whereas bacteria, protozoa, Valonia, myxomycetes, and other syncitial protophyta are noncellular, not unicellular.

The author then tells us that it is his aim to strip tissue culture of its former atmosphere of mystery and complication, of gray walls, black gowns, masks, hoods, etc., and huge witches' caldrons. But it was all these which made a visit to Carrel's laboratory so much fun. Furthermore, White introduces a witch's caldron the like of which Carrel never dreamed of. On page 70 is Shakespeare's witches' caldron from Macbeth (Act IV, Scene 1), which White proves is good tissue-culture soup. Shakespeare recommends the toad, and did not Ross Harrison use "lymph of frog?" "Ravin'd salt" surely is a Ringer or Locke-Lewis nutrient! I wondered how White could ever find an analogy for "Finger of birth-strangled babe." He did, by stating that sometimes it is customary to use human placental cord serum ("It can be purchased from Difco," i.e. the placental serum, not the strangled babe).

I am very much in favor of writing science with a personal touch. So is Dr. Otto Loewi, as he tells us in a delightful prefatory chapter in the current Annals of Physiological Reviews. But how far can an author introduce his character into a scientific treatise? At least we can say this for White, his good humor enlivens what could easily have been a dull handbook, and the quotations, from Theophrastus in Greek to Haberlandt in German, do not detract from the excellence and accuracy of the nearly two hundred pages devoted to technique.

Further pleasure is to be had, by those of us who grew up with the science of tissue culture, from two pages of portraits, including an excellent one of the author. But one misses some eminent names. White does full justice to the great ones now living; among them Harrison, the Lewises, Fischer, Gautheret, and Honor B. Fell, yet there are several omissions from the historically great, the most extraordinary of all being Strangeways. Miss Fell is now director of the laboratory which he founded. Strangeways introduced tissue-culture into England. Many able English investigators, e.g. Canti, were his students. I should prefer Strangeways' story of the living sausages

to Shakespeare's caldron, for there was truth as well as life in those sausages. To prove his theory that a pig could die and yet live, Strangeways often went to the country market and purchased a dozen fresh sausages made from pigs butchered the day before. "Never," he said, "have I failed to get a good culture of thriving cells from these sausages. The pig lives on."

Another omission, surely an oversight, is Levi. He played an outstanding part during the early years of tissue-culture work, and was Italy's foremost representative. These were leaders in the field, and some of those mentioned by White are not.

The pages devoted to methods, materials, solutions, and technique in general are complete to the last detail. Only a graduate of Hopkins would miss some mention of Shive's three salt solution by a fellow alumnus.

I presume every author has the right to emphasize his own work and discoveries. If he does not, then who will: but at times, it seems to me, the emphasis here is unnecessarily obvious. White claims credit for being the "first to establish continuous cultures of plant organs," and the "first to develop fully defined maintenance nutrients for both plant and animal cell." Robbins might well question the first priority, and a number of workers will question the second.

The "simple techniques for beginners" could be simpler. Calcium pantothenate, biotin, naphthalene acetic acid, chick plasma, horse serum, and a Carrel flask are not necessary for simple and fairly satisfactory cultures made by a beginner. A clean cover slip and slide, a saline solution and embryo extract are sufficient.

The Cultivation of Animal and Plant Cells is an interesting, a very "human," and thoroughly helpful manual by an expert. Perhaps its best feature is its reliability. Errors do not exist.

There is a philosophical lesson to be derived from White's manual. One hears so often that a method in experimental work, or a way of looking at things, even an entire field of science, is now a dead subject. Tissue culture was said to have died with Carrel and the thirty year old strain which he started in 1912. Actually, the method is, and always was, a good one. Today it is enjoying a remarkable rejuvenescence and solving problems varying from nutrition to the mechanism of protoplasmic streaming.—William Seifriz.

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